

METABOLISM OF
CARBON COMPOUNDS
IN THE C₄
MESOPHYLL CELL

a study with
Digitaria sanguinalis mesophyll
protoplasts and chloroplasts

Mats Hallberg



Lund 1983



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A study with *Digitaria sanguinalis*
mesophyll protoplasts and chloroplasts

by

Fil.kand. Mats Hallberg (Hb)

AKADEMISK AVHANDLING

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This summary is based on the following papers which will be referred to in the text by their Roman numerals:

- I Compartmentation and export of $^{14}\text{CO}_2$ fixation products in mesophyll protoplasts from the C_4 plant *Digitaria sanguinalis*.
Mats Hallberg and Christer Larsson
Arch. Biochem. Biophys. 208, 121-130 (1981)
- II Compartmentation and export of $^{14}\text{CO}_2$ fixation products in mesophyll protoplasts from the C_4 plant *Digitaria sanguinalis*.
Mats Hallberg and Christer Larsson
In: Photosynthesis IV. Regulation of Carbon Metabolism (Akoyunoglou, G., ed.), pp. 581-590, Balaban Intern. Science Services, Philadelphia, PA. (1981)
- III Highly purified intact chloroplasts from mesophyll protoplasts of the C_4 plant *Digitaria sanguinalis*. Inhibition of phosphoglycerate reduction by orthophosphate and by phosphoenolpyruvate.
Mats Hallberg and Christer Larsson
Physiol. Plant. 57, 330-338 (1983)
- IV Metabolism of $[1-^{14}\text{C}]$ 3-phosphoglycerate with mesophyll protoplasts and purified mesophyll chloroplasts from the C_4 plant *Digitaria sanguinalis*. Formation of hexose phosphates and starch.
Mats Hallberg and Christer Larsson
Manuscript, 32p. (1983)

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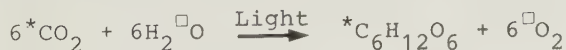
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1. INTRODUCTION

The capture and use of solar energy by photosynthetic organisms to convert carbon dioxide (CO_2) into carbohydrates and other cellular constituents, is a fundamental biochemical process upon which all other living organisms on earth depend. In the first part of this process light quanta are absorbed by pigments, such as chlorophylls and carotenoids in the green plants, and the energy is used to excite electrons. These electrons pass through a chain of electron carriers, whereby the light energy is transformed into chemical energy in the form of an energy rich compound - adenosine triphosphate (ATP) - and reducing equivalents - reduced nicotinamide adenine dinucleotide phosphate (NADPH). In the second part of this process the chemical energy is used to reduce CO_2 to carbohydrates

($\text{C}_n\text{H}_{2n}\text{O}_n$), in a cyclic process involving carboxylation of a 5-carbon acceptor compound, formation of carbohydrates, and regeneration of the acceptor compound.

The electrons used to convert light energy to chemical energy are derived from water which is decomposed into oxygen and protons, giving the following over all reaction for photosynthetic carbon reduction:



where the source and fate of the carbon and oxygen are denoted. The oxygen in the atmosphere is derived from this reaction and can be regarded as a biproduct of photosynthesis. The production of carbohydrates and oxygen by photosynthetic organisms made the evolution of hetero-

trophic organisms, including man, possible. For our existence we extract energy from organic matter by oxygen-dependent oxidation of these compounds resulting in the production of CO_2 and water, the substrates of photosynthesis.

Fixation of carbon dioxide

In higher plants the photosynthetic process takes place in chloroplasts, cell organelles present in all green tissue. The green colour comes from the light-absorbing chlorophyll molecules. The components of the primary reactions involved in the conversion of light energy to chemical energy are located in special structures, the thylakoid membranes, while the fixation of CO_2 takes place in the soluble part of the chloroplast, the stroma. The chloroplast is bounded by a double membrane, and every compound that is taken up by or exported from the chloroplast must pass this barrier, actively or passively.

The C_3 pathway of photosynthesis

With the discovery of ^{14}C (Ruben and Kamen 1940) came the tool necessary for the mapping of the CO_2 fixation pathway. Calvin, Benson and coworkers used $^{14}\text{CO}_2$ to follow the path of carbon during carboxylation and photosynthetic carbon reduction. By 1954 they had formulated the complete reaction cycle with the 3-carbon compound 3-phosphoglycerate as the first stable product after the carboxylation of the acceptor molecule ribulose-1,5-biphosphate (Benson and Calvin 1948, Bassham et al. 1954). The cycle is alternatively named the Calvin cycle, the Calvin-Benson cycle, the reductive pentose phosphate cycle or the C_3 pathway.

The C₄ pathway

Among the early products of CO₂ fixation were also the 4-carbon compounds malate and succinate. These were first thought to be part of the regenerative phase of the Calvin cycle. Later it was found that certain tropical grasses incorporated ¹⁴CO₂ mainly into the C₄ acids malate and aspartate, before the label appeared in 3-phosphoglycerate and other Calvin cycle intermediates (Kortschak et al. 1965, Hatch and Slack 1966). Hatch and Slack (1970) formulated a new pathway in which phosphoenolpyruvate carboxylase catalyzed the incorporation of CO₂ into oxaloacetate. This C₄ acid was reduced to malate or aminated to aspartate before CO₂ was released and refixed in the Calvin cycle. These early studies also implicated a possible compartmentation of the reactions between two different cell types, with the C₄ acid formation and conversion to malate and aspartate taking place predominantly in the mesophyll cells, and the Calvin cycle located predominantly in the bundle sheath cells (Björkman and Gauhl 1969, Slack et al. 1969).

Already in 1884 Haberlandt observed that certain species of grass had distinct cell layers surrounding the vascular bundle, an outer layer of mesophyll cells with few chloroplasts and an inner layer of thick-walled bundle sheath cells containing numerous chloroplasts. He named this anatomical feature "Kranz leaf anatomy" and speculated on the possible division of labour between these different types of cells.

The evolutionary and adaptational advantages for plants with "Kranz anatomy" and C₄ photosynthesis were revealed

from studies on the ecological adaptation of certain plants with high photosynthetic rates (Björkman 1974). These plants were found in semiarid regions and in saline habitats where high light intensities, high temperatures, and water deficits were prevailing. Some of the characteristic features of plants with high photosynthetic rates were: low CO_2 compensation point, hyperbolic response of photosynthesis to increasing light intensity, ability to withstand high salt levels and low water potentials in the soil, and low rates of photorespiration.

It is now accepted that plants with "Kranz anatomy" and with high rates of photosynthesis fix CO_2 in the mesophyll cells via phosphoenolpyruvate carboxylase forming C_4 acids. The C_4 acids are transported to the bundle sheath cells where they are decarboxylated and the released CO_2 is re-fixed via the Calvin cycle in the bundle sheath chloroplasts. The 3-carbon compounds remaining after decarboxylation of the C_4 acids, are transported back to the mesophyll cells to complete the cycle. This new metabolic route was named the C_4 pathway. A scheme of the general flow of fixed carbon in the C_4 plant and the reactions taking place in the different cell types is given in Fig.1 (redrawn from Hatch and Osmond (1976)).

The role of the mesophyll cell in C_4 photosynthesis

The intracellular localization of the reactions of the C_4 pathway have been studied using mechanically prepared mesophyll and bundle sheath cell fractions. These studies provided the basis for the formulation of the complete reaction scheme (reviewed by Hatch and Osmond 1976).

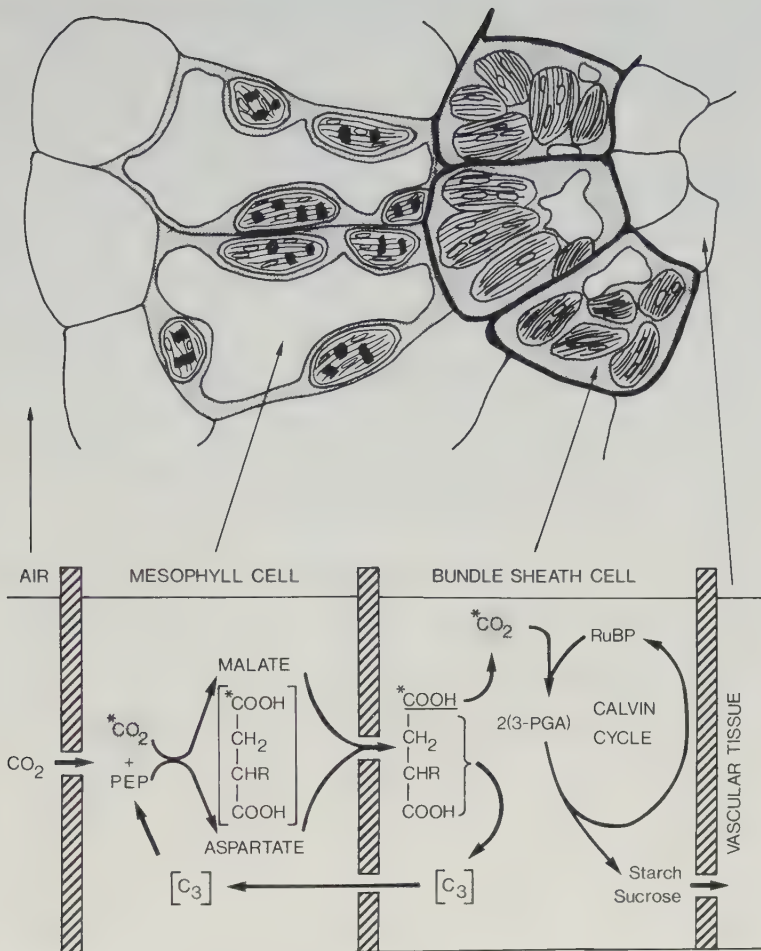


Fig. 1. A schematic representation of C₄ photosynthesis with the main reactions and their intercellular localization. The morphological relationship of the different cell types is shown in the drawing of a cross section of a grass leaf (NADP-malic enzyme type). PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglycerate; RuBP, ribulose-1,5-bisphosphate; C₃, 3-carbon compound. (Redrawn from Hatch and Osmond (1976)).

Studies with isolated mesophyll protoplasts and bundle sheath strands have ascertained and extended this scheme (Gutierrez et al. 1975). Hatch et al. (1975) divided the C₄ plants into three types, the NADP-malic enzyme type, the

NAD-malic enzyme type and the phosphoenolpyruvate carboxy-kinase type, based on the mechanisms for decarboxylation of C_4 acids in the bundle sheath cells from various species.

In mesophyll cells from C_4 plants of the NADP-malic enzyme type, oxaloacetate is mainly converted to malate in the chloroplasts by NADP-malate dehydrogenase, and malate is exported to the bundle sheath cells. The mesophyll chloroplasts also contain aspartate aminotransferase which can aminate oxaloacetate to aspartate, but aspartate is not considered a major transport metabolite in this type of C_4 plant. In the mesophyll cells from C_4 plants of the NAD-malic enzyme type and the phosphoenolpyruvate carboxy-kinase type, oxaloacetate is aminated to aspartate in the cytoplasm and aspartate is exported to the bundle sheath cells. The CO_2 acceptor molecule, phosphoenolpyruvate, is formed in the chloroplasts from pyruvate by pyruvate- P_i -dikinase, an enzyme unique to C_4 plants. Apart from enzymes involved in the C_4 pathway the mesophyll chloroplasts also contain the enzymes of the Calvin cycle for converting 3-phosphoglycerate to dihydroxyacetone phosphate, namely 3-phosphoglycerate kinase, NADP:glyceraldehyde-3-phosphate dehydrogenase and triose phosphate isomerase. These reactions, together with their energy demand are summarized in Fig. 2.

The bundle sheath chloroplasts of the NADP-malic enzyme type C_4 plant contain low amounts of stacked membrane regions (grana thylakoids), and have low rates of photosystem II activity (Black and Mollenhauer 1971, Mayne et al. 1974). The low rates of photosystem II activity in

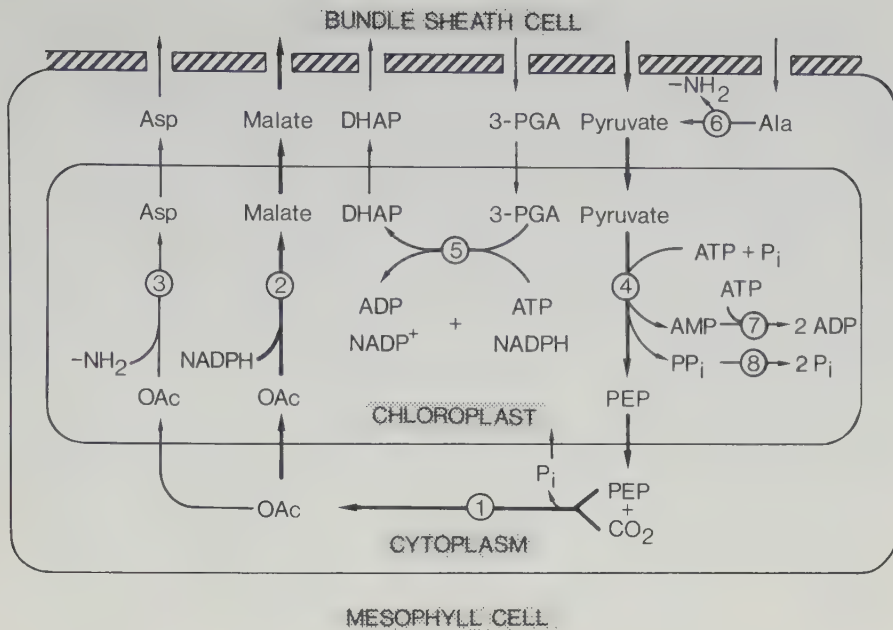


Fig. 2. Reactions taking place in the mesophyll cell of C₄ plants of the NADP-malic enzyme type showing the intracellular localization and the energy demand of the reactions. Heavy arrows show the main flow of carbon in this type of mesophyll cell. The enzymes catalyzing the reactions are: 1 phosphoenolpyruvate carboxylase; 2 NADP-malate dehydrogenase; 3 aspartate aminotransferase; 4 pyruvate-P_i-dikinase; 5 3-phosphoglycerate kinase, NADP: glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase; 6 alanine aminotransferase; 7 adenylate kinase; 8 pyrophosphatase. Abbreviations used: Asp, aspartate; PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; Ala, alanine. (Redrawn from Hatch and Osmond (1976)).

the bundle sheath chloroplasts leads to a NADPH deficit in the Calvin cycle. The decarboxylation of malate by the NADP-malic enzyme can supply half of the NADPH required for CO₂ fixation in the bundle sheath chloroplasts. The reduction of 3-phosphoglycerate to dihydroxyacetone phosphate in the mesophyll chloroplasts can supply the rest of the NADPH via a shuttle of 3-phosphoglycerate and dihydroxyacetone phosphate between the two cell types (Fig. 2).

The current scheme for the reactions in C_4 photosynthesis taking place in the mesophyll cell (see Fig. 2) requires a rapid transport of several compounds in and out of the mesophyll cell and chloroplast. The flow of metabolites between mesophyll and bundle sheath cells has been proposed to be driven by concentration gradients through pores in the cell wall, the so called plasmodesmata (Osmond and Smith 1976).

In chloroplasts of C_3 plants, the transport of inorganic phosphate and phosphorylated 3-carbon compounds across the envelope is mediated by a phosphate translocator, and the transport of dicarboxylic acids by a dicarboxylate translocator (Fliege et al. 1978). Similar translocators have also been found in mesophyll chloroplasts from C_4 plants. A major difference between the phosphate translocator of C_3 and C_4 mesophyll chloroplasts is that phosphoenolpyruvate is transported with a much lower K_m on the phosphate translocator of C_4 mesophyll chloroplasts compared to C_3 chloroplasts (Huber and Edwards 1977a,b, Day and Hatch 1981a,b). This could be expected since phosphoenolpyruvate is a major transport metabolite in C_4 mesophyll chloroplasts but not in C_3 chloroplasts.

The plant used in this study, *Digitaria sanguinalis*, is of the NADP-malic enzyme type and is thought to export mainly malate from the mesophyll to the bundle sheath cells in exchange for pyruvate. Furthermore, the mesophyll cells of this C_4 plant import 3-phosphoglycerate, which is reduced to dihydroxyacetone phosphate and this product is exported back to the bundle sheath cells. The enzymes needed for the reduction of 3-phosphoglycerate are part

of the Calvin cycle, and these enzymes are present both in mesophyll and bundle sheath chloroplasts. However, no other enzymes of the Calvin cycle have been detected in mesophyll chloroplasts of C_4 plants. Starch is a product of C_3 photosynthesis, and is stored as grains inside the chloroplasts. Morphological studies with C_4 plants have revealed the presence of starch grains both in bundle sheath and mesophyll chloroplasts (Black and Mollenhauer 1971, Laetsch 1974). However, enzymes for starch formation from triose phosphates have not been found in mesophyll chloroplasts, or have been proportional to the contamination by bundle sheath material in the mesophyll chloroplast preparations (Edwards and Black 1971, Hatch and Kagawa 1973, Downton and Hawker 1973).

2. AIMS OF THE PRESENT WORK

The predicted flow of metabolites in and out of the mesophyll cells can either be symplastic through plasmodesmata and driven by concentration gradients, or it could be a transport across the plasma membrane mediated by special translocator proteins. In order to investigate the latter possibility we considered it necessary to obtain pure and intact mesophyll protoplasts. Such a preparation was developed in Paper I. With the preparation of protoplasts we assume that the plasmodesmata are lost. The possibility of a regulated transport of C_4 metabolites across the plasma membrane was investigated by using these purified mesophyll protoplasts. In this context we studied the pattern of labelled products obtained after incorporation of $^{14}CO_2$ in the light using isolated mesophyll protoplasts

and different 3-carbon compounds as precursors. The intracellular compartmentation and the export of these products have been of particular interest (Paper I and II).

Another question regarding the predicted flow of metabolites concerns the transport of phosphorylated carbon compounds across the mesophyll chloroplast envelope. Is phosphoenolpyruvate transported on a separate translocator in exchange for inorganic phosphate, or are inorganic phosphate, phosphoenolpyruvate, 3-phosphoglycerate and dihydroxyacetone phosphate transported on the same phosphate translocator, as is the case with C_3 chloroplasts? The properties of the phosphate translocator can be studied indirectly by measuring 3-phosphoglycerate-dependent oxygen evolution with isolated mesophyll chloroplasts, and record the inhibitory effect of different compounds that may compete with 3-phosphoglycerate for uptake via the translocator. For this purpose we prepared highly intact mesophyll chloroplasts with low cytoplasmic contamination. A low cytoplasmic contamination was essential to avoid metabolism of the added compounds outside the chloroplasts (Paper III).

These purified chloroplasts were also used for studies on the metabolism of 3-phosphoglycerate. We tried to answer the question: can mesophyll chloroplasts metabolize triose phosphates to hexose phosphates and further to starch? The products of metabolism of ^{14}C -labelled 3-phosphoglycerate were determined as well as the activities of the enzymes needed for formation of starch from triose phosphates (Paper IV).

3. PLANT MATERIAL

The plant used throughout this study was *Digitaria sanguinalis* (L) Scop., a grass species of the Poaceae family, showing the typical "Kranz leaf anatomy" with a single layer of thick-walled bundle sheath cells surrounding the vascular bundle. Larger mesophyll cells surround these cells in a cylindrical arrangement (Downton and Tregunna 1968, Black and Mollenhauer 1971).

Plants were grown from seeds obtained from the Botanical Garden, Lund, in standard potting soil in a growth chamber. Light was from dysprosium lamps giving a light intensity of 14 000 to 20 000 lux and a quantum flux density of 230 to 400 $\mu\text{mol per m}^2$ and s, and with a light/dark cycle of 16h/8h. The temperature was 30°C during the light period and fell to 25°C during the dark period. The plants were watered every third day with tap water.

Under these growth conditions plants could be harvested after 16 days. Usually the two oldest leaves of 16 to 18-day plants were used. The first 2 cm at the base of the leaves were not used in the preparation of the mesophyll material, since the cells at the base are not differentiated into mesophyll and bundle sheath cells. The leaves are growing from the base and differentiation of the cells occurs as they elongate and mature along the leaf axis. The chlorophyll content of the isolated mesophyll protoplasts (Paper I) was in the same range as that of protoplasts from plants grown under natural light supplemented with artificial light (Edwards and Black 1971).

4. PREPARATION PROCEDURES

In order to study the distribution of enzymatic reactions between mesophyll and bundle sheath cells, an isolation procedure providing efficient separation between the cell types was needed. Based on the fact that bundle sheath cells have thicker cell walls, Björkman and Gauhl (1969) developed a differential grinding technique to produce mesophyll and bundle sheath cell extracts. A similar method involving differential grinding and filtration, was used by Edwards and Black (1971) to prepare mesophyll cells and bundle sheath strands from *Digitaria sanguinalis*. However, this mechanical method yielded cell preparations that were contaminated with intracellular material such as broken organelles and cytosolic enzymes originating from cells broken during the relatively harsh grinding treatment.

Isolation and purification of mesophyll protoplasts

In 1973 Kanai and Edwards reported on the first successful application of the protoplast isolation technique on C_4 plant leaves. Mesophyll protoplasts were released from leaf tissue by treatment with cell wall degrading enzymes. Huber and Edwards (1975c) extended the method to a number of C_3 and C_4 grasses.

We prepared mesophyll protoplasts from *Digitaria sanguinalis* leaf segments using a 2 hour digestion at 30°C in an enzymatic medium with 2% Driselase (Kyowa Hakko Co. Ltd. Tokyo, Japan), and otherwise according to Huber and Edwards (1975c).

Phase partition

When the water soluble polymers Dextran T 500 and polyethylene glycol (PEG) 4000 are mixed above certain concentrations they form two immiscible phases with a lower dextran-rich phase and an upper PEG-rich phase. Such two-phase systems contain up to 90% water and can be made suitable for biological material by the addition of an osmoticum (sucrose, sorbitol), buffers and salts. Membrane vesicles, organelles and cells often distribute preferentially to one of the bulk phases and the interface. Surface properties, such as charge and hydrophobicity, are the main factors determining the partition behaviour of a given particle.

Polymer-polymer two-phase systems and their properties have been described by Albertsson (1971) and their application in the purification of biological material have been reviewed by Larsson and Andersson (1979), Albertsson et al. (1982) and Larsson (1983). Separation of different materials can be achieved either by a multi-step counter-current distribution (CCD) technique (Albertsson 1971) for analytical purposes, or for preparative use, by simple batch procedures involving only a few partition steps.

Kanai and Edwards (1973) were the first ones to use phase partition for purification of protoplasts. They prepared protoplasts from C_3 , C_4 and CAM plants in a phase system composed of Dextran T 40 and PEG 6000 at 2°C. In the present work (Paper I-IV) protoplasts from *Digitaria sanguinalis* were purified in a Dextran T 500-PEG 4000 phase system. From work with spinach chloroplast preparations it was known

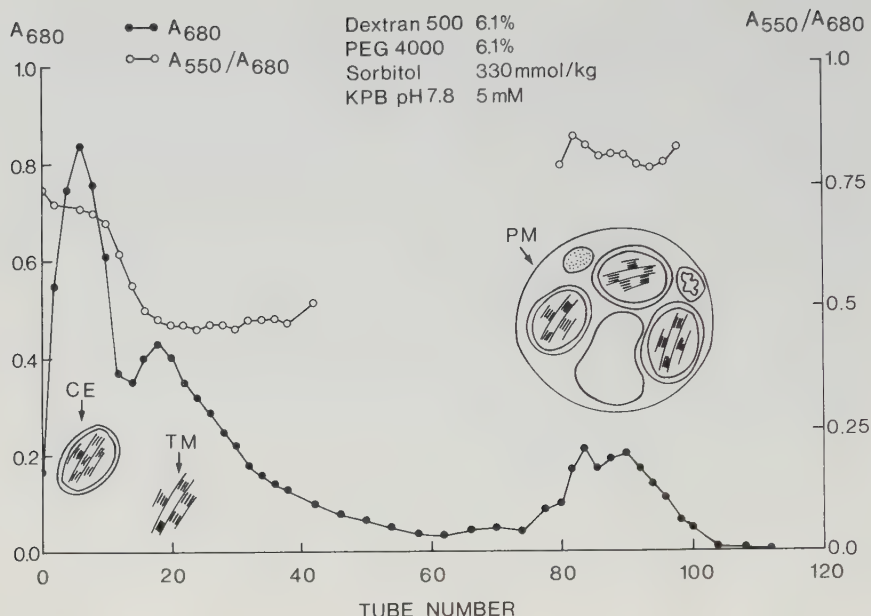


Fig. 3. Counter current distribution (CCD) of a chloroplast preparation from *Digitaria* mesophyll protoplasts. An automatic apparatus (Albertsson 1971) was used and 112 transfers were made. The chlorophyll content, absorbance at 680 nm, and the intactness of the particles, ratio of absorbance at 550 to absorbance at 680 nm (Karlstrom and Albertsson 1970), are given. The drawings show the content of each peak and which membrane surface the different particles expose to the phase system. KPB, potassium phosphate buffer; CE, chloroplast envelope; TM, thylakoid membrane; PM, plasma membrane.

that chloroplasts containing particles surrounded by the plasma membrane, so called multiorganelle complexes, had a high partition coefficient, i.e. partitioned mainly to the upper phase in a phase system with these polymers (Larsson et al. 1971).

In a preliminary experiment we tried to purify mesophyll chloroplasts prepared from mesophyll protoplasts, by CCD at 4°C in a phase system with Dextran T 500 and PEG 4000 (Fig.3).

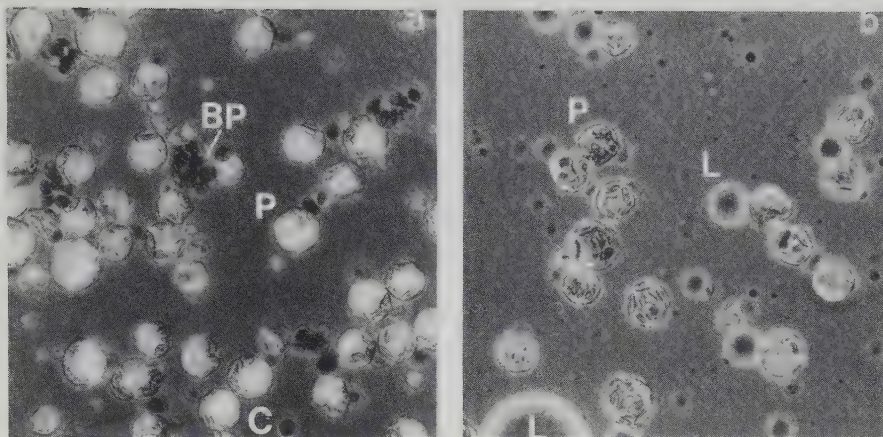


Fig. 4. Phase contrast light micrographs of *Digitaria* mesophyll protoplasts. a, protoplasts after differential centrifugation. Intact protoplasts (P) appear bright against the dark background (the dye Evans Blue was included in the medium). Note broken protoplasts (BP) and free chloroplasts (C). b, purified protoplasts suspended in the upper phase obtained after phase partition. Note intact protoplasts (P) and droplets of the lower phase (L). The average protoplast diameter was 17 μm .

Intact and broken chloroplasts partitioned to the lower phase and were collected in the beginning of the CCD train. Remaining intact protoplasts were found at the end of the CCD train, indicating a high partition ratio for this plasma membrane-bounded particle. We developed a batch procedure for purifying mesophyll protoplasts, taking advantage of the high partition ratio of these particles (Paper I). The purification was performed at room temperature since *Digitaria* mesophyll protoplasts are inactivated at low temperatures (Huber and Edwards 1975 c). The quality of the preparation was checked in the light microscope and the intactness of the protoplasts was examined with the dye Evans Blue (Kanai and Edwards 1973). The dye, having a molecular weight of 961 and being negatively charged, is not supposed to penetrate an intact membrane. Intact protoplasts therefore exclude the dye

and appear brightly yellow against a blue background. A comparison of the protoplast preparation before and after phase partition (Fig. 4a,b) showed that contaminating broken protoplasts had been removed almost completely.

The protoplast suspension containing polymers was used directly for metabolic studies (Paper I and II) and as starting material for preparation of mesophyll chloroplasts (Paper III and IV).

Protoplast extracts

A protoplast extract (Gutierrez et al. 1975, Huber and Edwards 1975a) was produced by passing the protoplast suspension through a Hamilton syringe thereby breaking the protoplasts but leaving most of the chloroplasts intact. This was done immediately before use, and the extract was kept on ice during sampling. This extract contained chloroplasts suspended in a dilute cytoplasm and metabolized added substrates without the transport restriction of the plasma membrane. The extract was used in $^{14}\text{CO}_2$ fixation experiments as a comparison to intact protoplasts (Paper I) and in studies with intact chloroplasts (Paper III and IV) as a fraction representing fresh unpurified chloroplasts.

Preparation of intact mesophyll chloroplasts

Purified protoplasts are excellent to use as starting material for the preparation of intact chloroplasts, since only mild forces are required to disrupt the plasma membrane. The most simple and common method for preparing mesophyll chloroplasts involves the breaking of protoplasts by passing them several times through a finely meshed nylon net followed

by collection of the chloroplasts by a single low-speed centrifugation (Huber and Edwards 1975a,b, Edwards et al. 1979, Day et al. 1981). Although these chloroplasts are highly intact and metabolically active, they are contaminated with cytosolic components. Thus, they contain 2 to 5% of the total phosphoenolpyruvate carboxylase activity present in the protoplasts, and even up to 25% of some other cytoplasmic enzymes (Huber and Edwards 1975b). Most of the earlier conclusions on enzyme location and the metabolism of carbon compounds by C_4 mesophyll chloroplasts are based on results obtained with such rather impure chloroplasts. We considered it necessary to obtain a more well-defined chloroplast preparation to be able to perform a more accurate study of the metabolism and enzyme content of mesophyll chloroplasts.

We prepared mesophyll chloroplasts from mesophyll protoplasts purified by phase partition. The protoplast suspension was passed several times through a 20 μ m nylon net to disrupt the protoplasts, and the released chloroplasts were collected by differential centrifugation. Remaining small protoplasts were removed from the chloroplast preparation by phase partition in a system similar to that used for purification of protoplasts, but at 4°C. The chloroplast preparation still contained broken chloroplasts, and these could not be removed by phase partition since intact and broken chloroplasts have a similar partition behaviour (see Fig. 3). Instead, intact chloroplasts were separated from broken ones by density gradient centrifugation using Percoll. The purified chloroplasts were stored on ice since they are less stable at room temperature (Huber and Edwards 1975c).

5. TRANSPORT IN MESOPHYLL PROTOPLASTS

Regulation of C_4 acid formation in mesophyll protoplasts and protoplast extracts

In the current model for C_4 photosynthesis as outlined in Fig. 2, the mesophyll cells import 3-carbon compounds from the bundle sheath cells and convert these compounds into phosphoenolpyruvate, which is the substrate for CO_2 fixation in mesophyll cells. In C_4 plants of the NADP-malic enzyme type such as *Digitaria sanguinalis*, pyruvate is regarded as the main 3-carbon compound returning from the bundle sheath cells. The enzyme content of the mesophyll cells also allows alanine and 3-phosphoglycerate to act as precursors for CO_2 fixation (Hatch and Osmond 1976). Alanine can be converted to pyruvate in the cytoplasm by alanine aminotransferase, a conversion that demands the presence of an amino-group acceptor such as 2-oxoglutarate. The mesophyll cells can convert 3-phosphoglycerate into phosphoenolpyruvate by the action of the glycolytic enzymes phosphoglyceromutase and enolase, enzymes that are present in *Digitaria* mesophyll cells (Huber and Edwards 1975b).

Huber and Edwards (1975a) reported on the pattern of C_4 acid formation in *Digitaria* mesophyll protoplasts and protoplast extracts using pyruvate, 3-phosphoglycerate, and alanine plus 2-oxoglutarate as substrates for $^{14}CO_2$ fixation. They found a similar labelling pattern with all these substrates, with malate as the major C_4 acid formed and with most of the incorporated ^{14}C in the C_4 acids oxaloacetate, malate and aspartate. The rates of $^{14}CO_2$ fixation with protoplast extracts were about five times higher than

with intact protoplasts. With phosphoenolpyruvate as substrate they obtained high rates of $^{14}\text{CO}_2$ fixation with oxaloacetate as the main product, both in the dark and in the light.

We have repeated some of the $^{14}\text{CO}_2$ fixation experiments of Huber and Edwards (1975a,b), using isolated mesophyll protoplasts and protoplast extracts (Paper I).

Intact protoplasts fixed $^{14}\text{CO}_2$ at low rates, and protoplast extracts at higher rates when pyruvate, 3-phosphoglycerate, or alanine plus 2-oxoglutarate were given as substrates. Malate was the major labelled C_4 acid when pyruvate was used as substrate, whereas oxaloacetate was the major $^{14}\text{CO}_2$ fixation product with 3-phosphoglycerate as substrate. The reduction of oxaloacetate to malate occurs inside the mesophyll chloroplasts at the expense of NADPH (Fig.2). The mesophyll chloroplasts can reduce 3-phosphoglycerate to dihydroxyacetone phosphate, a reduction consuming NADPH as well as ATP. The pattern of labelled C_4 acids obtained with pyruvate and 3-phosphoglycerate as substrates for $^{14}\text{CO}_2$ fixation, indicates a competition for available NADPH between oxaloacetate and 3-phosphoglycerate reduction. This competition has later been observed also with maize mesophyll chloroplasts (Day and Hatch 1981).

The labelling of C_4 acids with alanine plus 2-oxoglutarate as substrates for $^{14}\text{CO}_2$ fixation resembled the pattern observed with pyruvate as substrate except that the label in aspartate had increased at the expense of oxaloacetate (Paper I). The increased label in aspartate with alanine present indicated a need for external amino

groups if aspartate was to become a major labelled product.

Protoplasts and protoplast extracts incorporated $^{14}\text{CO}_2$ at high rates with phosphoenolpyruvate, or phosphoenolpyruvate plus glutamate as substrates. The main C_4 acid formed was oxaloacetate, but with glutamate present label in aspartate increased considerably (Paper I).

Our results are largely in agreement with those of Huber and Edwards (1975a). However, in our hands the differences between the $^{14}\text{CO}_2$ fixation rates obtained with protoplasts and protoplast extracts, as well as the differences in the pattern of labelled C_4 acids, were very much dependent on the 3-carbon compound supplied as substrate. The composition of 3-carbon precursors imported from the bundle sheath cells may thus partly determine the composition of C_4 acids exported from the mesophyll cells.

Compartmentation and export of $^{14}\text{CO}_2$ fixation products with mesophyll protoplasts

The current view, that transport of 3- and 4-carbon compounds between mesophyll and bundle sheath cells occurs via symplastic transport through plasmodesmata, is based mainly on theoretical considerations. Hatch and Osmond (1976) calculated that the flux of C_4 acids from mesophyll cells to bundle sheath cells in maize were about 3 times the flux required to sustain intact leaf photosynthesis, assuming a maximum C_4 acid concentration of 38 mM in the cytoplasm, and assuming that the transport was via diffusion of C_4 acids through the central part of the plasmodesmata pores.

An alternative to the transport through the plasmodesmata is the apoplastic transport route. This route would be through the plasma membrane of the mesophyll cell, via the water space in the cell walls and through the plasma membrane of the bundle sheath cell. However, this route is not considered to play an important role since the movement of ions in general through the plasma membrane and the cell wall is slow (Hatch and Osmond 1976). On the other hand, if the transport through the plasma membrane was facilitated by special transport proteins the apoplastic route may be important.

We used isolated mesophyll protoplasts to assess the role of the plasma membrane in the uptake and export of carbon compounds during $^{14}\text{CO}_2$ fixation (Paper I and II). We also tried to estimate the pools of the C_4 acids in the chloroplast and cytoplasm compartments of intact protoplasts performing CO_2 fixation (Paper II).

Intact mesophyll protoplasts fixed $^{14}\text{CO}_2$ at low rates with pyruvate, 3-phosphoglycerate, and alanine plus 2-oxoglutarate as substrates. Most of the labelled products were found outside the protoplasts with malate as the main product, except when 3-phosphoglycerate was given as substrate which gave oxaloacetate as the major export compound (Paper I and II). Phosphoenolpyruvate induced high rates of $^{14}\text{CO}_2$ fixation with 98% of the label in exported C_4 acids, mainly oxaloacetate. When glutamate was present together with phosphoenolpyruvate, aspartate was a major product found outside the protoplasts.

The results from the measurements on pool sizes of C_4 acids after 6 min of $^{14}CO_2$ fixation indicated that the intraprotoplast concentration of malate was rather independent of the rate of $^{14}CO_2$ fixation and of malate export from the protoplasts. The intraprotoplast concentration of aspartate depended on these factors in a way similar to malate although the concentration increased somewhat with increasing export rate. We therefore proposed that the export of malate and aspartate across the plasma membrane was regulated with internal pool sizes that were independent of export rates (paper I). Oxaloacetate, however, was exported at rates directly proportional to the intraprotoplast concentration, indicating a movement through the plasma membrane by simple diffusion.

In Paper II kinetic measurements on the chloroplast and cytoplasm pools of malate, aspartate and oxaloacetate were performed as time-course studies of $^{14}CO_2$ fixation. We developed a method whereby the chloroplast and cytoplasm pools of C_4 acids could be measured separately together with export from protoplasts. Such measurements indicated that the cytoplasm pool of malate was relatively independent of the 3-carbon compound used as substrate for $^{14}CO_2$ fixation. The concentration of malate in the cytoplasm was around 4 mM after 14 min of $^{14}CO_2$ fixation and was still increasing at that time. The chloroplast pool of malate, on the other hand, was more dependent on the substrate used and the concentration was lower than in the cytoplasm.

The aspartate pools were difficult to measure due to rapid turnover of oxaloacetate to aspartate during the

course of extraction and sample treatment. This made it impossible to obtain reliable data on the chloroplast and cytoplasm pools of aspartate. Only when alanine plus 2-oxoglutarate was used as substrate for $^{14}\text{CO}_2$ fixation this problem was not encountered. With this substrate, the values for the cytoplasm pool of aspartate in the time-course study were similar to those of malate. Thus, the time-course experiments indicated that the cytoplasm pool of malate was relatively independent of the export rate of malate, which supported the suggestion that there was a regulated transport of malate across the plasma membrane.

Malate should be the major C_4 acid formed during CO_2 fixation in mesophyll cells of NADP-malic enzyme type plants, and malate export should match the rate of CO_2 fixation observed with intact leaves of 200-400 μmol per mg chlorophyll and hour. However, the rates of malate export in the experiments described above were one order of magnitude lower than the expected rates. High export rates of C_4 acids were only obtained with phosphoenolpyruvate as substrate and in that case oxaloacetate, and not malate, was the major export product. We suggested that this could be due to low rates of non-cyclic electron transport, and thus low rates of oxaloacetate reduction, in the intact protoplasts.

All of the conclusions, in Paper I and II, regarding pool sizes and export rates from protoplasts are based on the assumption that a given substrate passes the plasma membrane, undergoes metabolic conversions inside the protoplasts, and that products found in the medium have been exported from intact protoplasts. However, more recent ob-

servations indicate that our protoplast preparations are contaminated with free phosphoenolpyruvate carboxylase. The high rates of oxaloacetate "export" with phosphoenolpyruvate as substrate may therefore be due to $^{14}\text{CO}_2$ fixation occurring in the medium outside the protoplasts. The observed "export" of malate and aspartate may similarly be explained by a few percent of broken protoplasts, giving free intact chloroplasts in the assay medium. Together with the presence of cytoplasmic enzymes and phosphoenolpyruvate carboxylase in the medium this would lead to oxaloacetate, malate and aspartate formation without participation of intact protoplasts - the "exported" products would have been formed in the medium. If this was the case the observed intraprotoplast pools may be due to a slow uptake of external substrate and/or endogenous substrate.

Therefore, in order to assess the true metabolic capacity of intact protoplasts, and the role of the plasma membrane in the intercellular transport, the measurements should be improved by:

- a) finding assay conditions that inhibit the external phosphoenolpyruvate carboxylase and metabolism by free chloroplasts, without disturbing the metabolism by the intact protoplasts
- b) speeding up the fractionation of protoplasts using silicon oil centrifugation technique (Robinson and Walker 1979, Wirtz et al. 1980), to avoid turnover of metabolites during fractionation. This method also allows for the correction of external medium present in the pellet fractions (Heldt

1980)

c) preparing plasma membrane vesicles for transport studies.

If plasma membranes were prepared also from leaves one may exclude the possibility that the cell-wall degrading enzymes used for protoplast preparation have altered the properties of the plasma membrane.

Thus the relative importance of transport via carriers in the plasma membrane and the proposed symplastic diffusion through plasmodesmata still remains unclear.

6. THE PHOSPHATE TRANSLOCATOR OF C_4 MESOPHYLL CHLOROPLASTS

The conversion of pyruvate to phosphoenolpyruvate, and of 3-phosphoglycerate to dihydroxyacetone phosphate in the mesophyll chloroplasts are two of the main reactions in C_4 photosynthesis (Fig. 2). The localization of these reactions to the chloroplast stroma demand that a rapid transport of phosphorylated compounds occur across the chloroplast envelope.

Metabolite exchange studies with *Digitaria* mesophyll chloroplasts have indicated the presence of a phosphate translocator transporting phosphoenolpyruvate in exchange for inorganic phosphate and 3-phosphoglycerate (Huber and Edwards 1977b). Similar studies with maize mesophyll chloroplasts also indicated the presence of a phosphate translocator, transporting inorganic phosphate, 3-phosphoglycerate, dihydroxyacetone phosphate and phosphoenolpyruvate with similar K_m (Day and Hatch 1981). This phosphate translocator is similar to the one found in the envelope of C_3 chloroplasts, except that the latter transports phosphoenolpyruvate with a K_m one order of magnitude higher than the K_m obtained with the other phosphorylated metabolites mentioned above (Fliege et al. 1978).

The reduction of 3-phosphoglycerate to triose phosphate in the mesophyll chloroplast consumes 1 ATP and 1 NADPH per molecule of 3-phosphoglycerate reduced, and is thereby coupled to non-cyclic electron transport and oxygen evolution. The competition for the phosphate translocator between different phosphorylated carbon compounds can be studied by inhibiting 3-phosphoglycerate-dependent oxygen evolution

with different possible transport compounds. Such studies showed that phosphoenolpyruvate was not competing with 3-phosphoglycerate for the phosphate translocator, whereas inorganic phosphate was (Edwards et al. 1979, Day et al. 1981). These results are in conflict with those obtained in studies on metabolite exchange (Huber and Edwards 1977b, Day and Hatch 1981), which suggested that phosphoenolpyruvate, 3-phosphoglycerate and inorganic phosphate shared a common carrier.

We used isolated *Digitaria* mesophyll chloroplasts to study their substrate-dependent oxygen evolution capacity and the properties of the phosphate translocator (Paper III).

Both pyruvate and oxaloacetate stimulated 3-phosphoglycerate-dependent oxygen evolution with isolated chloroplasts. Pyruvate may stimulate by uncoupling non-cyclic electron transport, since pyruvate is converted to phosphoenolpyruvate inside the chloroplasts under ATP consumption (Day et al. 1981). Oxaloacetate may stimulate oxygen evolution by being reduced to malate during consumption of NADPH, thereby increasing non-cyclic electron transport. Since both pyruvate and oxaloacetate stimulated oxygen evolution, these measurements indicated that 3-phosphoglycerate reduction was limited neither by the supply of ATP, nor by the supply of NADPH. We concluded that the rate limiting step in these chloroplasts was the transport of 3-phosphoglycerate across the envelope via the phosphate translocator. If this was the case, the stimulation of 3-phosphoglycerate-dependent oxygen evolution by pyruvate

could rather be due to an increased uptake of 3-phosphoglycerate via the translocator in exchange for phosphoenolpyruvate, which had been formed inside the chloroplasts from the added pyruvate.

The rates of 3-phosphoglycerate-dependent oxygen evolution obtained with isolated mesophyll chloroplasts were lower than the rates obtained with protoplast extracts (Paper III) and also lower than the rates predicted from photosynthetic rates of intact leaves. These low rates probably depended on a low translocator activity limiting 3-phosphoglycerate uptake. The low translocator activity may be due to low levels of exchangeable phosphorylated compounds inside the isolated chloroplasts. The chloroplasts may have lost inorganic phosphate by leakage during the time of preparation. The facts that the inner envelope membrane of C_4 mesophyll chloroplasts have a very large surface (Laetsh 1974), and that already freshly prepared mesophyll chloroplasts have low endogenous metabolite levels (Day and Hatch 1981a, Huber and Edwards 1977a,b) support this suggestion.

We studied the affinity of the phosphate translocator for phosphoenolpyruvate by adding increasing amounts of this metabolite to isolated *Digitaria* mesophyll chloroplasts during 3-phosphoglycerate-dependent oxygen evolution (Paper III). Phosphoenolpyruvate and inorganic phosphate inhibited this reaction to about the same extent in isolated chloroplasts, indicating that phosphoenolpyruvate was transported with a K_m similar to inorganic phosphate. In contrast to the results with isolated chloro-

plasts, 3-phosphoglycerate-dependent oxygen evolution with protoplast extracts was stimulated by phosphoenolpyruvate at low concentrations and only inhibited at high concentrations. Inorganic phosphate, however, inhibited this reaction with protoplast extracts in a manner similar to that observed with isolated chloroplasts (Paper III).

The stimulatory effect of phosphoenolpyruvate on 3-phosphoglycerate-dependent oxygen evolution with protoplast extracts may be explained by the presence of cytoplasmic enzymes. Either phosphoenolpyruvate was carboxylated to oxaloacetate by phosphoenolpyruvate carboxylase, or it was converted to pyruvate by pyruvate kinase. Oxaloacetate or pyruvate would then in turn stimulate 3-phosphoglycerate-dependent oxygen evolution, as discussed above, thereby masking the inhibitory effect of phosphoenolpyruvate. The reason for the conflicting results in the literature concerning the effect of phosphoenolpyruvate on 3-phosphoglycerate-dependent oxygen evolution on the one hand and on 3-phosphoglycerate uptake on the other hand could be that the chloroplasts used were impure and contained contaminating cytoplasmic enzymes, which metabolized the added phosphoenolpyruvate. Our results support the view that in C_4 mesophyll chloroplasts inorganic phosphate, 3-phosphoglycerate, dihydroxyacetone phosphate and phosphoenolpyruvate are transported with similar K_m and on the same carrier.

7. METABOLISM OF 3-PHOSPHOGLYCERATE AND ENZYMES FOR STARCH SYNTHESIS

In the current model for C_4 photosynthesis the mesophyll chloroplasts contain enzymes for conversion of 3-phosphoglycerate to dihydroxyacetone phosphate but they are not considered to contain any other enzymes of the Calvin cycle, or any of the enzymes needed for synthesis of starch from triose phosphates (Hatch and Osmond 1976). The function of phosphoglycerate reduction to dihydroxyacetone phosphate in the mesophyll chloroplasts from C_4 plants of the NADP-malic enzyme type, would be to supply the photosystem II deficient bundle sheath chloroplasts with ATP and NADPH via exchange of 3-phosphoglycerate and dihydroxyacetone phosphate. Kagawa and Hatch (1974) studied the metabolism of ^{14}C -labelled 3-phosphoglycerate with maize mesophyll chloroplasts and they found dihydroxyacetone phosphate as the main product. Label in other Calvin cycle intermediates and in starch was negligible.

The synthesis of starch from triose phosphates involves aldolase, fructose-1,6-bisphosphatase, hexose phosphate isomerase, ADP-glucose pyrophosphorylase and starch synthetase. No conclusive evidence on the presence of these enzymes in C_4 mesophyll chloroplasts has been published (see Introduction).

We have used purified *Digitaria* mesophyll chloroplasts (prepared according to Paper III) and mesophyll protoplast extracts to reinvestigate the metabolism of ^{14}C -labelled 3-phosphoglycerate (Paper IV). Labelled 3-phosphoglycerate was mainly converted into dihydroxyacetone phosphate with

isolated chloroplasts and protoplast extracts, but label was also found in hexose phosphates and in starch. Most of the labelled dihydroxyacetone phosphate was exported from the chloroplasts.

Protoplast extracts also metabolized labelled 3-phosphoglycerate through the CO_2 fixation pathway forming labelled oxaloacetate, malate and aspartate. 3-phosphoglycerate can be converted to phosphoenolpyruvate by the cytoplasmic enzymes phosphoglucomutase and enolase, and thereby act as substrate for CO_2 fixation (compare Paper I). This route of 3-phosphoglycerate metabolism constituted 20% of the total rate in protoplast extracts. The reduction of oxaloacetate to malate can compete with 3-phosphoglycerate reduction for available NADPH as seen in $^{14}\text{CO}_2$ fixation experiments (Paper I and II). Conversion of labelled 3-phosphoglycerate with protoplast extracts was inhibited by 30 to 70% by the addition of pyruvate (Paper IV). Pyruvate induced high rates of CO_2 fixation and oxaloacetate reduction, as seen from parallel $^{14}\text{CO}_2$ fixation experiments. These rates of oxaloacetate reduction decreased the label in 3-phosphoglycerate reduction products by 40 to 80%, whereas label in CO_2 fixation products was decreased by about 20%. This competitive effect of oxaloacetate reduction was also observed with intact protoplasts (Paper II).

The label in starch in our experiments corresponded to rates of hexoses incorporation of 0.02 to 0.1 μmol hexose per mg chlorophyll and hour (Paper IV and unpublished). These rates are much lower than the rates of starch formation in spinach chloroplasts (0.5 to 5 μmol hexose incorporated per mg chlorophyll and hour; Heldt et al. 1977)

Since the C_4 mesophyll chloroplasts appeared to lack enzymes for starch synthesis from triose phosphates, Hatch and Osmond (1976) proposed that the starch found in mesophyll chloroplasts could have been formed from imported hexoses or hexose phosphates originating from the bundle sheath cells. No evidence of transport of hexoses or hexose phosphates into mesophyll chloroplasts has been presented.

We obtained low activities of aldolase and fructose-1,6-bisphosphatase with the isolated mesophyll chloroplasts as compared to the activities measured with spinach chloroplasts (C_3 chloroplasts containing the Calvin cycle and with a high capacity to form starch from 3-phosphoglycerate) (Paper IV). However, the capacity of the mesophyll chloroplasts to form ADP-glucose from hexose phosphates involving hexose phosphate isomerase, phosphoglucomutase and ADP-glucose pyrophosphorylase, was equal to or higher than in spinach chloroplasts (Paper IV). These results support the suggestion made by Hatch and Osmond (1976) that imported hexoses or hexose phosphates may be the main substrates for starch synthesis in mesophyll chloroplasts.

By comparing the activities per mg chlorophyll of these enzymes in intact protoplasts with those in isolated chloroplasts we could show that one third to half of the activity of aldolase, fructose-1,6-bisphosphatase, hexose phosphate isomerase and phosphoglucomutase was confined to the chloroplasts; the rest of the activity would be cytoplasmic (Paper IV). The activity per mg chlorophyll of ADP-glucose pyrophosphorylase in mesophyll chloroplasts and protoplasts was similar, indicating that this enzyme was confined to the

chloroplasts.

The limiting step in starch formation according to the enzyme studies was the formation of fructose-1,6-bisphosphate from triose phosphates catalyzed by aldolase. The aldolase activity corresponded to 1,6 μmol hexose formed per mg chlorophyll and hour (Paper IV), a rate one order of magnitude higher than the measured rate of incorporation of labelled 3-phosphoglycerate into starch.

In the current model for C_4 photosynthesis the mesophyll chloroplasts carry out some of the major primary reactions of CO_2 fixation and C_4 acid conversion. The bundle sheath chloroplasts contain the enzymes of the Calvin cycle and carry out the secondary CO_2 fixation and concomitant starch formation. Our results indicate that the mesophyll chloroplasts have a low capacity for formation of hexose phosphates from triose phosphates, but contain high activities of enzymes needed for starch formation from hexose phosphates. Thus, in a situation where bundle sheath chloroplasts are loaded with starch, they may export hexoses or hexose phosphates to the mesophyll chloroplasts. These hexoses may be converted to starch at high rates according to the measured enzyme complement if isolated mesophyll chloroplasts, yielding the observed starch deposit in these chloroplasts.

Further studies on the ability of the mesophyll chloroplasts to take up hexoses or hexose phosphates are needed to establish this route of starch synthesis.

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Compartmentation and Export of $^{14}\text{CO}_2$ Fixation Products in Mesophyll Protoplasts from the C_4 -Plant *Digitaria sanguinalis*

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Isolated mesophyll protoplasts, and protoplast extracts containing intact chloroplasts, from the C_4 species *Digitaria sanguinalis* have been used to study compartmentation and export of C_4 acids, using different C_3 precursors as substrate for $^{14}\text{CO}_2$ fixation. Mg^{2+} was necessary for maximum $^{14}\text{CO}_2$ fixation rates with both protoplasts and protoplast extracts, whereas Mg^{2+} was inhibitory for oxaloacetate and phosphoglycerate reduction. This inhibition could be overcome by preincubating the materials in the light with excess of EDTA before addition of Mg^{2+} . Under these conditions pyruvate as substrate for $^{14}\text{CO}_2$ fixation induced mainly malate formation, whereas phosphoglycerate as substrate induced oxaloacetate formation, indicating competition for available NADPH between oxaloacetate and phosphoglycerate reduction. Oxaloacetate could be exported from the protoplasts at rates comparable to the rates of $^{14}\text{CO}_2$ fixation in intact leaves ($200 \mu\text{mol}/\text{mg Chl} \times \text{h}$). This product probably passed the plasma membrane by simple diffusion, whereas the export of malate and aspartate seemed to be regulated, with the size of the intraprotoplast pool being relatively independent of the export rate. It is concluded that transport via the plasma membrane-cell wall path may play a role in metabolite flow during photosynthesis in C_4 plants.

In plants having the C_4 pathway of photosynthesis, coordinated function of two different cell types is necessary. The atmospheric CO_2 is fixed in the mesophyll cells by carboxylation of PEP,¹ and then transported as a C_4 dicarboxylic acid to the bundle sheath cells. In these cells the C_4 acids are decarboxylated and the released CO_2 is refixed via the Calvin cycle. Suitable C_3 precursors for regeneration of PEP are shuttled back to the mesophyll cells to complete the cycle (for review see (1)).

The distribution of enzymes between the

two cell types is well documented (1, 2), and metabolic studies with isolated mesophyll cells and protoplasts (3, 4), and with bundle sheath strands (5, 6) show that these can perform the expected reactions. However, the flow of and compartmentation of metabolites according to the suggested pathways are not well established.

Generally, transport across the plasma membranes and the cell wall is thought to be too slow to account for the rapid flux of intermediates that are needed. Instead, transport via the numerous plasmodesmata (symplastic transport), which are seen to connect the cell types in electron micrographs (7), is regarded as the main path (1, 8).

The aim of this work was to study compartmentation and export of C_4 -acids, using isolated mesophyll protoplasts, and protoplast extracts, from the C_4 -plant *Digitaria sanguinalis*—a plant belonging to the NADP-malic enzyme group (9). With the isolation of protoplasts the plasmodes-

¹ Abbreviations used: PEP, phosphoenolpyruvate; BSA, bovine serum albumin; PEG, polyethylene glycol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; Chl, chlorophyll; PGA, 3-phosphoglycerate; OAc, oxaloacetate; Rib-5-P, ribose-5-phosphate; OxG, 2-oxoglutarate; medium A, 0.5 M sorbitol, 1 mM MgCl_2 , 1 mM KH_2PO_4 , 0.1% BSA (fatty acid free), adjusted to pH 6.0 with KOH; medium B, 0.5 M sorbitol, 5 mM potassium phosphate buffer, pH 7.8, and 0.1% BSA.

desmata are lost. Excretion of products from isolated protoplasts should therefore be due to transport across the plasma membrane. The results are discussed in relation to current views on metabolite flow in C_4 plants.

MATERIALS AND METHODS

Chemicals. Dextran 500, batch No. 3447, was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Polyethylene glycol 4000 (Carbowax 4000) was purchased from Union Carbide, New York. Catran (1-methyl-2-phenyl-ethylhydrazine-HCl) was a gift from Draco AB, Lund. $\text{NaH}^{14}\text{CO}_3$ (60 Ci/mol) was from The Radiochemical Centre, Amersham.

Plant culture. Crabgrass (*Digitaria sanguinalis* (L.) Scop.) was grown in standard potting soil in a growth chamber provided with dysprosium lamps (Osram HQIL, 400 W) giving a light intensity of 14,000 to 20,000 lx and a quantum flux density of 230 to 400 $\mu\text{E m}^{-2} \text{s}^{-1}$ between 400 and 700 nm. The light period was 16 h and the day/night temperature was 30/25°C. Leaves from plants 18 to 24 days old were used for the experiments, usually the two oldest leaves.

Isolation of mesophyll protoplasts. Leaves were harvested 1 h after the beginning of the light period and immediately cut in 0.7-mm-wide segments with a mechanical leaf cutter (10). The segments were collected in 50 ml of medium A: 0.5 M sorbitol, 1 mM MgCl_2 , 1 mM KH_2PO_4 , 0.1% BSA (fatty acid free), adjusted to pH 6.0 with KOH. The suspension was poured onto two layers of 80- μm nylon cloth, and the segments were washed twice in 50 ml of medium A to remove cellular material liberated during the cutting of the leaves.

A total of 8 g of washed segments was divided into two lots, and each lot was suspended in 200 ml of an enzymatic digestion medium: 2% Driselase (Kyowa Hakko Co. Ltd., Tokyo, Japan), 0.5 M sorbitol, 1 mM MgCl_2 , 1 mM KH_2PO_4 , 0.1% BSA, adjusted to pH 5.5 with KOH (the medium was clarified by a 20-min centrifugation at 7000g prior to use). The suspended segments were vacuum infiltrated enough to sink them. The enzymatic digestion was performed at 30°C on a waterbath with continuous shaking during the first half-hour, and gentle swirling of the solution every half-hour during the following 1.5 h.

Digested leaf segments were collected on four layers of 80- μm nylon cloth by gently pouring the suspension onto the cloth. Protoplasts were released by washing the segments four times with 50 ml of medium A. After each washing, segments, epidermal and vascular tissue were collected by filtering the suspension through four layers of 80- μm nylon cloth. The pooled filtrates, containing intact protoplasts

and intact and broken chloroplasts, were centrifuged for 2 min at 200g. The pellet was suspended in 35 ml of medium B: 0.5 M sorbitol, 5 mM potassium phosphate buffer, pH 7.8, and 0.1% BSA. The protoplasts were spun down at 200g for 1 min in a swing-out centrifuge, and resuspended in 3 ml of medium B.

Purification of protoplasts. Protoplasts were purified by partition in an aqueous two-phase system containing: 7.0% (w/w) Dextran 500, 7.0% (w/w) PEG 4000, 0.5 M sorbitol, 5 mM potassium phosphate, pH 7.8, and 0.1% BSA. A 12-g phase mixture was made in a Sorvall SS-34 centrifugal tube by mixing: 5.250 g of 20% (w/w) Dextran 500, 2.625 g of 40% (w/w) PEG 4000, 3.000 g of a sorbitol solution containing 2 mol/kg, 0.300 g of 0.2 M potassium phosphate, pH 7.8, 0.012 g BSA, and finally 0.813 g of distilled water. After adding protoplasts equal to 3–4 mg of chlorophyll, suspended in 3 ml of medium B, a 15-g phase system with the above final composition was obtained. The tube was sealed with parafilm and inverted 20 times to mix the system. The phases were then allowed to settle for 10 min at unit gravity. The upper phase was withdrawn with a pipet, taking care not to remove material from the interface. The collected upper phase was repartitioned with 5.8 g of fresh lower phase from a bulk system of the same composition. The second upper phase, containing purified protoplasts, was collected and diluted with an equal volume of 0.16 M sorbitol, 0.10 M Hepes, 0.1% BSA, adjusted to pH 7.6 with KOH, to give the final concentrations of the assay solution except for the polymers present. The intactness of the protoplasts was checked in the light microscope with the dye Evans blue (11).

The protoplast suspension, 10 ml with 100–150 μg Chl/ml, was stored at room temperature for further use. All operations during isolation and purification of protoplasts were carried out at 22°C, since these protoplasts are inactivated at cold room temperature (10).

Protoplast extracts. Protoplast extracts (3) were prepared immediately before use by passing the suspended protoplasts through a 100- μl Hamilton syringe, producing an extract with about 80% intact chloroplasts (phase-contrast microscopy).

$^{14}\text{CO}_2$ fixation. All $^{14}\text{CO}_2$ fixation assays were run in a medium with the final composition of 0.33 M sorbitol, 50 mM Hepes-KOH, pH 7.6, 2 mM EDTA, 3 mM MgCl_2 , 2 mM KH_2PO_4 , 0.1% BSA, 3.4 mM $\text{NaH}^{14}\text{CO}_3$, and 50–100 μg Chl/ml. Other substrates than $\text{NaH}^{14}\text{CO}_3$ were added as indicated in figures and tables. The requirement of both protoplasts and protoplast extracts for MgCl_2 and KH_2PO_4 for maximum $^{14}\text{CO}_2$ fixation rates was adopted from Huber and Edwards (3) and the requirement of both materials for Mg^{2+} confirmed by us. Assays were run in test tubes incubated on a waterbath at 30°C, with slow

back and forth movement of the tubes perpendicular to the light path. Sedimenting particles were resuspended regularly. Light was provided by a Philips Photolita KM PF 215, 375-W lamp, giving a total quantum flux of $2500 \mu\text{E m}^{-2} \text{s}^{-1}$ between 400 and 700 nm.

After 2 min preillumination of the sample in a medium without MgCl_2 and $\text{NaH}^{14}\text{CO}_3$, these compounds were added to start $^{14}\text{CO}_2$ fixation (see results, Figs. 1 and 2).

For end-point measurements the samples (100 μl) were incubated for 6 min after addition of MgCl_2 and $\text{NaH}^{14}\text{CO}_3$, and then killed with 50 μl of 0.6% Catran in 1 M HCl.

For kinetic studies, 10- μl samples were withdrawn at intervals and spotted on small filter papers wetted with 0.6% Catran in 1 M HCl. The filter papers were dried and the radioactivity was determined by liquid scintillation counting.

For studies on export of products from protoplasts (or from chloroplasts with protoplast extracts), 90 μl of the 100- μl reaction mixture was transferred to a 1-ml centrifugal tube, and the protoplasts were

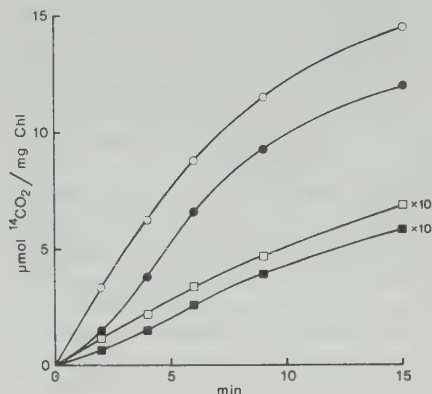


FIG. 2. Pyruvate dependent $^{14}\text{CO}_2$ fixation with intact protoplasts (squares) and protoplast extracts (circles). The samples were either preilluminated for 2 min with 2 mM EDTA in the medium before addition of 3 mM Mg^{2+} and $\text{NaH}^{14}\text{CO}_3$ at time zero (open symbols); or the reaction medium was complete at the onset of light (solid symbols).

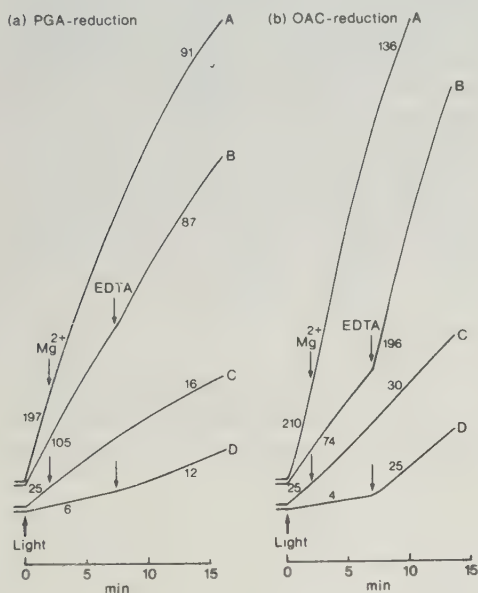


FIG. 1. (a) PGA-reduction, and (b) OAc-reduction with intact protoplasts (C, D), and protoplast extracts (A, B). The figures at the oxygen traces give the rates as μmol substrate reduced/ $\text{mg Chl} \times \text{h}$. In A and C the medium contained 2 mM EDTA and no Mg^{2+} at the onset of light; and 3 mM Mg^{2+} was added after 2 min as indicated. In B and D the medium contained 1 mM Mg^{2+} added in the dark; and 3 mM EDTA was added after 7 min in the light.

sedimented by a short acceleration in a Beckman Microfuge B (or when the sample was a protoplast extract, the chloroplasts were sedimented by a 5-s centrifugation). From the supernatant, 70 μl was added to 35 μl of 0.6% Catran in 1 M HCl, and subsequently 50 μl of 0.2% Catran in 0.33 M HCl was added to the drained pellet.

Total incorporation of ^{14}C was determined by liquid scintillation counting on 5- or 10- μl samples from each fraction.

Analysis of $^{14}\text{CO}_2$ fixation products. Soluble products of $^{14}\text{CO}_2$ fixation, including Catran derivatives of ketoacids, were separated by thin-layer electrophoresis for 40 min, using conditions according to Schürmann (12), followed by autoradiography. Radioactive spots were scraped off and the radioactivity was determined by liquid scintillation counting. Products were identified by running authentic compounds in the same system.

PGA and OAc reduction. Reduction of PGA and OAc with protoplasts and protoplast extracts was assayed as substrate-dependent O_2 evolution under saturating light. Measurements were made at 30°C in a total volume of 1 ml, containing 7–10 $\mu\text{g Chl}$, in a medium with 0.33 M sorbitol, 50 mM Hepes-KOH, pH = 7.6, 2 mM KH_2PO_4 , and 0.1% BSA. A Clark-type oxygen electrode (Hansatech Ltd., King's Lynn, Norfolk) was used. Substrate concentrations were: 2 mM PGA and 4 mM OAc. Other additions as indicated in figures. The light source was a slide projector giving a quantum flux density of $2500 \mu\text{E m}^{-2} \text{s}^{-1}$ between 400 and 700 nm.

Chlorophyll determination. Chlorophyll was estimated by the method of Arnon (13).

RESULTS AND DISCUSSION

Partition of Protoplasts

It was necessary to purify the protoplast preparation since it also contained intact and broken chloroplasts, some intact cells, and fiber elements. Partition in aqueous polymer two-phase systems has been used earlier to purify protoplasts. Kanai and Edwards (11) used a phase system where protoplasts collected at the interface after a short centrifugation of the system. However, it is known that the plasma membrane, which is the outer limitation of a protoplast, has a high partition coefficient in two-phase systems containing the polymers Dextran 500 and polyethylene glycol 4000 (14). In the same system chloroplasts and cells partition to the interface. Taking advantage of the preference of the protoplasts for the polyethylene glycol-rich upper phase, we could purify them with the simple two-step batch procedure outlined in the method section.

Light Activation and Mg^{2+}

Mg^{2+} inhibition of $^{14}CO_2$ fixation with isolated chloroplasts was first demonstrated by Avron and Gibbs (15). This phenomenon was later investigated by Huber (16–18) and Demmig and Gimmmler (19). Both Huber and Demmig and Gimmmler suggest that Mg^{2+} indirectly (without penetrating the chloroplast envelope) inhibits the light activation of some key enzymes in photosynthesis of both C_3 and C_4 chloroplasts.

We found inhibitory effects of Mg^{2+} also with our preparations of protoplasts and protoplast extracts. Since $^{14}CO_2$ fixation with mesophyll protoplasts or protoplast extracts, of *D. sanguinalis* cannot be run without Mg^{2+} in the medium (3), it was necessary to find assay conditions which allowed high rates not only of $^{14}CO_2$ fixation, but also of OAc and PGA reduction, as well as of PEP formation from pyruvate.

Effect of Mg^{2+} on OAc and PGA Reduction

Both OAc and PGA reduction were strongly inhibited already at a Mg^{2+} concentration of 1 mM, if the Mg^{2+} was added in the dark. This inhibition occurred both with protoplasts and protoplast extracts, and could be partly reversed by addition of 2 mM EDTA in excess during the light period (Fig. 1a, b: curves B and D). However, OAc reduction was not inhibited, and PGA reduction was only slightly inhibited if the Mg^{2+} (final concentration, 3 mM) was added after 2 min preillumination of the materials in the presence of 2 mM EDTA (Fig. 1a, b: curves A and C).

Mn^{2+} was tested as an alternative to Mg^{2+} , but the inhibitory effect of Mn^{2+} was even stronger (data not shown).

Conflicting results have been reported concerning the ability of mesophyll protoplast extracts, or isolated chloroplasts, to reduce OAc. Hatch and Kagawa (20) prepared maize mesophyll chloroplasts with OAc-reducing capacity (1 mM OAc, medium with 5 mM $Na_2P_2O_7$, 1 mM EDTA, and 2 mM $MgCl_2$). Huber and Edwards (3) failed to show OAc reduction with mesophyll protoplast extracts from *D. sanguinalis*, unless pyruvate was present (0.5 mM OAc, medium with 1 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$). The differences in results may be explained by the differences in media with respect to free divalent cations.

The Mg^{2+} inhibition of PGA reduction may involve the light activation of NADP-glyceraldehyde-3-P-dehydrogenase, as shown for barley chloroplasts by Huber (17).

Effect of Mg^{2+} on Phosphorylation of Pyruvate

To determine if the enzyme pyruvate- P_1 -dikinase, which catalyzes the light-dependent conversion of pyruvate to PEP, also was inhibited by Mg^{2+} added in the dark, we measured the pyruvate-dependent $^{14}CO_2$ fixation with protoplasts and protoplast extracts. As seen in Fig. 2, this reaction was not inhibited, but without preillumination a lag-phase was found.

As a consequence of the experiments described above, a preillumination period of 2 min in the absence of Mg^{2+} (and $NaH^{14}CO_3$) and with 2 mM EDTA, was used in the $^{14}CO_2$ fixation experiments. This procedure allowed maximum activities of all enzymes involved. Furthermore, Mg^{2+} was omitted from all media where it was not a necessary component.

Products of $^{14}CO_2$ Fixation

Incorporation of label into different products after 6 min of $^{14}CO_2$ fixation was determined for protoplasts and protoplast extracts using substrates according to Table I. Protoplast extracts fixed $^{14}CO_2$ at rates 1.4 to 33 times higher than those of intact protoplasts. For both materials 88 to 98% of the carbon incorporated was found in the three C_4 acids OAc, malate,

and aspartate. These results are similar to those obtained earlier by Huber and Edwards (3, 4).

Without substrate, or when alanine was supplied, protoplasts fixed $^{14}CO_2$ at low rates with malate and aspartate as main products. Ribose-5-P, a substrate for CO_2 fixation via the Calvin cycle, did not increase the rate, and the distribution of label resembled that obtained with alanine or no substrate added (Table I). This shows that alanine alone could not be used as a substrate, that there was a low endogenous rate of $^{14}CO_2$ fixation, and that no functional bundle sheath cells were present.

Intact protoplasts fixed $^{14}CO_2$ mainly into malate when they were supplied with pyruvate as substrate (Table IA). With PGA, on the other hand, OAc became the major product, indicating competition for

TABLE I
PRODUCTS OF $^{14}CO_2$ FIXATION

(A) Distribution of label after 6 min of $^{14}CO_2$ fixation with mesophyll protoplasts ^a								
Product	Substrate ^b							
	Pyruvate	PGA	Ala + OxG	Alanine	None	Rib-5-P	PEP	PEP + Glu
Malate	55.7	16.8	47.0	52.4	54.6	48.5	4.9	3.5
Aspartate	10.0	18.5	17.8	39.3	36.9	41.1	0.8	15.9
OAc	30.2	62.7	31.9	0.6	0.5	1.4	87.8	77.6
Others	4.1	2.0	3.3	7.7	8.0	9.0	6.5	3.0
$^{14}CO_2$ fixation ($\mu\text{mol}/\text{mg Chl} \times \text{h}$)	5.9	4.9	2.6	0.53	0.50	0.54	243	216
(B) Distribution of label after 6 min of $^{14}CO_2$ fixation with mesophyll protoplast extracts ^a								
Product	Substrate ^b							
	Pyruvate	PGA	Ala + OxG	PEP	PEP + Glu			
Malate	89.1	9.4	77.8	12.6	9.2			
Aspartate	2.4	38.7	9.9	2.2	33.9			
OAc	6.8	49.4	9.8	81.3	53.5			
Others	1.8	2.5	2.4	3.9	3.4			
$^{14}CO_2$ fixation ($\mu\text{mol}/\text{mg Chl} \times \text{h}$)	57.6	15.9	11.1	373	307			

^a Figures are percentage of total fixed $^{14}CO_2$.

^b Substrate concentrations were: 10 mM pyruvate, 5 mM PGA, 10 mM Ala + 2 mM OxG, 10 mM Ala, 5 mM Rib-5-P, 10 mM PEP, 10 mM PEP + 2 mM Glu.

available NADPH between PGA and OAc reduction.

If alanine was combined with OxG, the $^{14}\text{CO}_2$ fixation rate with intact protoplasts became comparable to those obtained with pyruvate and PGA, as shown earlier by Huber and Edwards (4). The labeling pattern resembled that with pyruvate, but the fixed carbon in aspartate increased to 18% of total due to the amino nitrogen available from alanine.

The primary CO_2 fixation product in C_4 photosynthesis, OAc, was the main product when PEP was supplied as substrate to intact protoplasts (Table IA). When amino nitrogen was made available through the addition of 2 mM glutamate, the incorporation into aspartate increased from 1 to 16% of total ^{14}C .

The incorporation patterns with protoplast extracts showed the same general features as those of intact protoplasts (Table IB). However, OAc was metabolized to a greater extent with protoplast extracts than with protoplasts. Protoplast extracts behaved as if OAc produced in the medium (diluted cytoplasm) was more accessible for reduction or amination by chloroplasts, as compared to the situation with intact protoplasts.

Compartmentation of Products

In separate experiments, the distribution of label between the particles and the surrounding medium was determined for protoplasts and protoplast extracts (Table IIA, B). For both materials relatively less of the total label was found in the pellet fraction when the fixation rate was increased. Pellets from protoplast extracts (mainly chloroplasts) also contained relatively less of the fixed $^{14}\text{CO}_2$ than protoplast pellets. However, if the absolute values are compared the chloroplasts generally contained more label than the protoplasts. This may partly be explained by the higher fixation rates obtained with protoplast extracts, and partly by other differences between the two systems. Direct comparisons between pool sizes obtained with the two systems must therefore be made with some care.

When intact protoplasts were supplied with pyruvate, PGA, or alanine + OxG, relatively low rates of $^{14}\text{CO}_2$ fixation were induced, and the pellets contained mostly malate and aspartate and only low amounts of OAc (Table IIA). With PEP (or PEP + glutamate) as substrate, which induced high rates of $^{14}\text{CO}_2$ fixation, OAc became a major product inside the protoplasts.

Malate and OAc were the main products outside the protoplasts, when pyruvate or alanine + OxG were supplied. With PGA as substrate, protoplasts exported mainly OAc. OAc was the main product outside the protoplasts also with PEP as substrate, but the addition of glutamate increased the amount of aspartate from 1 to 20% of total export.

In protoplast extract pellets, malate and aspartate were the main products found, together constituting between 80 and 92% of the label when pyruvate, PGA, alanine + OxG, or PEP + glutamate were used as substrates (Table IIB). Only when PEP alone was supplied, OAc became a major product inside the chloroplasts.

The pool size of malate inside the chloroplasts was around or above the values obtained for the intraprotoplast pool, except when PGA was the substrate. In this case, competition between PGA and OAc for available NADPH led to low malate formation and a large pool of OAc. With PEP + glutamate, aspartate was produced at a high rate, and the chloroplast pool of OAc was kept low in spite of the high $^{14}\text{CO}_2$ fixation rate. Instead the aspartate pool became large. This is in agreement with the finding of Huber and Edwards (4), that aspartate aminotransferase is located inside the chloroplasts in NADP-malic enzyme species, in contrast to NAD-malic enzyme species and PEP-carboxykinase species, where this enzyme is found in the cytoplasm.

When PEP alone was supplied, the chloroplasts were saturated with OAc but the further metabolism was limited by available amino groups, and probably also by noncyclic electron transport. Thus, malate formation with pyruvate was higher than with PEP, although the $^{14}\text{CO}_2$ fixation rate

TABLE II
COMPARTMENTATION OF $^{14}\text{CO}_2$ FIXATION PRODUCTS

Product	Substrate ^b							
	Pyruvate		PGA		Ala + OxG		PEP	
	Sup ^c	Pellet ^c	Sup	Pellet	Sup	Pellet	Sup	Pellet
(A) Distribution of label after 6 min of $^{14}\text{CO}_2$ fixation with mesophyll protoplasts ^a								
Malate	350	52	55	41	69	38	1080	56
Aspartate	49	32	100	46	13	36	200	53
OAc	280	3	430	3	43	1	20800	250
Others (%)	(2.3)	—	—	—	(5.0)	(3.1)	(11.7)	—
Total	690	87	590	90	132	49	25000	356
(%)	(88.8)	(11.2)	(86.7)	(13.3)	(62.9)	(37.1)	(98.6)	(1.4)
$^{14}\text{CO}_2$ fixation ($\mu\text{mol}/\text{mg Chl} \times \text{h}$)	7.8		6.8		2.1		254	
								217
(B) Distribution of label after 6 min of $^{14}\text{CO}_2$ fixation with mesophyll protoplast extracts ^a								
Malate	8290	93	210	15	5510	94	5480	64
Aspartate	170	20	705	96	780	36	850	73
OAc	520	3	2680	25	170	2	29300	190
Others (%)	(3.5)	(5.3)	(8.6)	(2.2)	(5.9)	(5.8)	(7.8)	(15.4)
Total	9310	120	3940	140	6870	140	38600	390
(%)	(98.7)	(1.3)	(96.6)	(3.4)	(98.8)	(2.0)	(99.0)	(1.0)
$^{14}\text{CO}_2$ fixation ($\mu\text{mol}/\text{mg Chl} \times \text{h}$)	94.3		40.8		70.1		390	
								347

^a The radioactivity in different products is expressed as nmol/mg Chl in supernatant and pellet, respectively, except for "Others," which is given as percentage. Values for total amount are expressed both as nmol $^{14}\text{C}/\text{mg Chl}$, and as percentage of total fixed $^{14}\text{CO}_2$.

^b Substrate concentrations: see Table I.

^c Protoplasts, in (A), and chloroplasts, in (B), were separated from the assay solution by a short centrifugation before killing and analysis.

was much lower. This could be due to an uncoupling effect of pyruvate on noncyclic electron transport (4).

Metabolite Flow

If the plasma membrane was a simple diffusion barrier, the outflow of $^{14}\text{CO}_2$ fixation products should be linear with the concentration maintained inside the protoplasts. In Fig. 3 we have plotted the intraprotoplast pool sizes of malate, aspartate, and OAc against the amounts found outside after 6 min of $^{14}\text{CO}_2$ fixation (data obtained from Table IIA). As seen in Fig. 3a, there is a linear relationship between the amount of OAc excreted and the amount found in the pellet. For malate, on the other hand, the intraprotoplast pool was around 55 nmol/mg Chl which was independent of the rate of malate export, and thus also of the substrate used (Fig. 3b). Aspartate export and internal pool size showed a similar relation, although the intraprotoplast pool increased with increasing export.

We therefore conclude that the plasma membrane in our mesophyll protoplast preparations exerts little or no limitation on the outflow of OAc, whereas the outflow of malate and aspartate in some way is regulated, with the internal pool sizes of malate and aspartate being relatively independent of the export rates.

In the case of protoplast extracts the

situation is somewhat different. OAc was produced in the medium and then taken up by the chloroplasts, probably through the dicarboxylic acid translocator (21) in exchange for malate or aspartate. Therefore the pool size of OAc inside the chloroplasts depended both on the $^{14}\text{CO}_2$ fixation rate and the rates of malate and aspartate formation in the chloroplasts (Table IIB). As seen in Fig. 4, the export of malate was relatively proportional to the pool size inside the chloroplasts and this pool exceeded the pool in the intact protoplasts. Aspartate export was dependent on a larger pool inside the chloroplasts, and the relation between internal pool size and export was similar to that obtained with protoplasts (Fig. 3b). It can therefore not be excluded, that the behavior of the aspartate pool in the experiments with protoplasts reflects more the properties of the chloroplast envelope than of the plasma membrane.

GENERAL DISCUSSION

Digitaria sanguinalis is a C_4 plant of the NADP-malic enzyme type. Its mesophyll cells are therefore supposed to export mainly malate to the bundle sheath cells, where malate can be decarboxylated in the chloroplasts by NADP-malic enzyme, and the resulting pyruvate can then be shuttled back to the mesophyll cells as substrate for malate production. This cycle should be able to operate at rates comparable to the $^{14}\text{CO}_2$ fixation rates of the

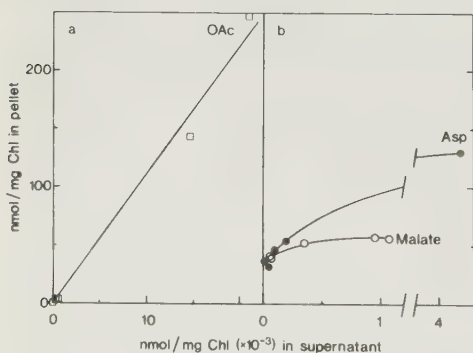


FIG. 3. (a, b) Plot of the intraprotoplast pool (pellet) versus total export (supernatant) after 6 min of $^{14}\text{CO}_2$ fixation with isolated intact protoplasts. Data are from Table IIA.

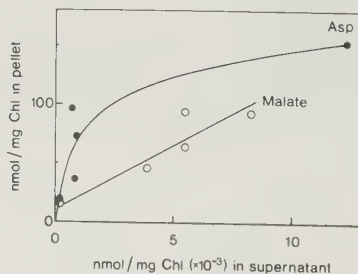


FIG. 4. Plot of the intrachloroplast pool (pellet) versus total export (supernatant) after 6 min of $^{14}\text{CO}_2$ fixation with a protoplast extract containing about 80% intact chloroplasts. Data are from Table IIB.

plant, that is 300–400 $\mu\text{mol } ^{14}\text{CO}_2/\text{mg Chl} \times \text{h}$ (22).

One would expect that isolated intact mesophyll protoplasts should be able to fix $^{14}\text{CO}_2$ and produce malate at very high rates when supplied with pyruvate and $\text{NaH}^{14}\text{CO}_3$. This is, however, not the case.

Huber and Edwards (3) found that the $^{14}\text{CO}_2$ fixation rates of protoplast extracts (broken protoplasts) were about five times those of intact protoplasts, and suggested that the plasma membrane limited the uptake of substrate. We found similar differences in $^{14}\text{CO}_2$ fixation rates, but in our hands the ratio between protoplast extract and protoplast activity was very much dependent on the substrate used (Tables I and II).

It has been shown that malate and aspartate can inhibit the PEP carboxylase activity in crude leaf extracts of *D. sanguinalis* (4). The low $^{14}\text{CO}_2$ fixation rates could therefore be caused by the build up of high concentrations of malate and aspartate in the cytoplasm, due to low export rates across the plasma membrane (23). However, from Table IIA it is evident that the malate and aspartate pools in the protoplasts differed little despite the large differences in $^{14}\text{CO}_2$ fixation rates obtained with, for instance, pyruvate and PEP. We rather believe that the concentration of PEP in the cytoplasm was limiting. Thus, the highest rates were obtained with PEP, while other substrates gave much lower rates, possible because each enzymatic and diffusive step needed to convert them to PEP successively lowered the concentration.

It could be argued that the rates obtained with protoplasts are due to a small percentage of broken protoplasts, and that the intact protoplasts are totally inactive. However, the apparent K_m for HCO_3^- has been found to be similar for isolated mesophyll cells and protoplasts of *D. sanguinalis*, and significantly larger than the K_m obtained with protoplast extract (3). This should not be the case if the activity obtained with protoplast preparations was due to contaminating broken protoplasts. There was also no indication of protoplast breakage during the experiments. If pro-

toplast breakage occurred this should result in increasing activities during a time-course experiment, but the rates were rather linear or even declining (Figs. 1 and 2).

$^{14}\text{CO}_2$ fixation products from the mesophyll cells are thought to be transported to the bundle sheath cells via plasmodesmata connecting the two cell types (symplastic transport), while direct transport across the plasma membranes and through the cell wall is regarded as too slow (1, 8). However, our results suggest that the plasma membrane of the mesophyll cells permits high rates of OAc export (about 200 $\mu\text{mol}/\text{mg Chl} \times \text{h}$, as calculated from Table IIA), and also relatively high rates of aspartate export (about 40 $\mu\text{mol}/\text{mg Chl} \times \text{h}$, Table IIA, PEP + Glu). The low rates of malate export which we obtained, seem rather to have been due to low rates of malate synthesis inside the protoplasts than to properties of the plasma membrane restricting the export.

This is suggested by the oxygen traces shown in Fig. 1b. Although the chloroplasts in the protoplast extract reduced OAc to malate at a rate exceeding 200 $\mu\text{mol}/\text{mg Chl} \times \text{h}$, the maximum rate obtained with intact protoplasts was only 30 $\mu\text{mol}/\text{mg Chl} \times \text{h}$. However, the $^{14}\text{CO}_2$ fixation experiments indicate that OAc can be transported across the plasma membrane at high rates, and that the intraprotoplast pool of OAc can be large without a high rate of malate production (Table IIA, PEP). Thus, the limiting step for OAc reduction in isolated protoplasts may be something else than substrate transport across the plasma membrane, e.g., limitations in noncyclic electron transport.

The high rates of OAc export and low rates of malate export which we have obtained with isolated mesophyll protoplasts are therefore probably an artifact, and due to the suppressed rate of OAc reduction. The situation *in situ* can be expected to be the opposite—that is high rate of malate export and low rate of OAc export. Our results suggest that this export *in situ* may occur via the plasma membrane-cell wall path. In fact, the relative indepen-

dence of malate and aspartate export on internal pool sizes (Fig. 3b) points to a regulated transport of these compounds across the plasma membrane of the mesophyll cells.

Isolated bundle sheath strands from several C_4 species can take up and decarboxylate malate and aspartate at high rates (5, 6). Under the assumption that the plasmodesmata are closed with the isolation of these cells, this is a further indication of transport via the plasma membrane-cell wall path, and would make the path from mesophyll to bundle sheath cells complete.

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COMPARTMENTATION AND EXPORT OF $^{14}\text{CO}_2$ FIXATION PRODUCTS IN MESOPHYLL
PROTOPLASTS FROM THE C_4 PLANT DIGITARIA SANGUINALIS

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ABSTRACT

Intact mesophyll protoplasts from the C_4 species *Digitaria sanguinalis* have been used to study compartmentation and export of C_4 acids, using different C_3 precursors as substrate for $^{14}\text{CO}_2$ fixation. Protoplasts were isolated by standard enzymatic procedures, and purified by partition in an aqueous dextran-polyethylene glycol two-phase system. To obtain maximal rates of oxaloacetate (OAc) and PGA reduction it was necessary to light activate the material in excess of EDTA before addition of Mg^{2+} . The $^{14}\text{CO}_2$ fixation rate became comparable to that of the intact leaf only when phosphoenolpyruvate (PEP) was supplied as substrate, giving OAc as the main export product (export rate $\leq 340 \mu\text{mol/mg Chl}\cdot\text{h}$). The rate of malate formation and export was low ($\leq 14 \mu\text{mol/mg Chl}\cdot\text{h}$) as compared to the rates expected from the proposed rôle of malate as the main transport compound in NADP-malic enzyme species. Only with available amino nitrogen (glutamate), aspartate became a major product and was exported at a rate of $\leq 85 \mu\text{mol/mg Chl}\cdot\text{h}$. The product pattern showed that OAc and PGA reduction competed for available NADPH. Thus, the composition of export products is determined by the substrate(s) used. PGA reduction was also investigated with ^{14}C -PGA. Kinetic studies on the compartmentation of C_4 acids between chloroplasts and cytoplasm were performed. Pool sizes (mM conc.) were calculated using compartment volume estimations from electronmicrographs. The cytoplasm pool of malate was independent of substrate and of export rate, whereas the OAc pool (protoplast) was directly proportional to the export rate. The aspartate pool resembled that of malate. This indicates a regulated transport across the plasma membrane of malate and aspartate, whereas OAc seems to diffuse out freely. We propose that transport via the plasma membrane - cell wall path plays an important role in metabolite flow during C_4 photosynthesis. This is in contrast to the generally held view of transport via plasmodesmata.

INTRODUCTION

In plants having the C_4 pathway of photosynthesis, coordinated function of two different cell types is necessary. The atmospheric CO_2 is fixed in the mesophyll cells by carboxylation of PEP, and then transported as a C_4 dicarboxylic acid to the bundle sheath cells. In these cells the C_4 acids are decarboxylated and the released CO_2 is refixed via the Calvin cycle. Suitable C_3 precursors for regeneration of PEP are shuttled back to the mesophyll cells to complete the cycle (c.f. 1).

The distribution of enzymes between the two cell types is well documented (1,2), but the flow of and compartmentation of metabolites according to the suggested pathways are not well established.

Generally, transport across the plasma membranes and the cell wall is thought to be too slow to account for the rapid flux of intermediates that are needed. Instead, transport via the numerous plasmodesmata (symplastic transport), which are seen to connect the cell types in electronmicrographs (3), is regarded as the main path (1,4).

The aim of this work was to study compartmentation and export of C_4 acids, using isolated mesophyll protoplasts from the C_4 plant *Digitaria sanguinalis* - a plant belonging to the NADP-malic enzyme group (5).

METHODS

Preparation of mesophyll protoplasts

Protoplasts were prepared by standard enzymatic procedures, and purified by partition in an aqueous polymer two-phase system (7.0% (w/w) dextran T 500, 7.0% (w/w) polyethylene glycol 4000, 0.5 M sorbitol, 5 mM potassium phosphate, pH 7.8, and 0.1% BSA. The upper phase, containing purified protoplasts, was collected and diluted to give the final concentrations of the assay solution except for the polymers present. The intactness of the protoplasts was checked with the dye Evans blue (6). The protoplast suspension (10 ml with 100-150 μ g Chl/ml) was stored at room temperature. All operations were carried out at 22 °C (except enzymatic digestion for 2h, which was at 30 °C), since these protoplasts are inactivated at cold room temperature (7).

$^{14}CO_2$ fixation

All $^{14}CO_2$ fixations assays were run at 30 °C in a medium with a final composition of 0.33 M sorbitol, 50 mM HEPES-KOH pH 7.6, 2 mM EDTA, 3 mM $MgCl_2$, 2 mM KH_2PO_4 , 0.1% BSA, 3.4 mM $NaH^{14}CO_3$. Other substrates were added as indicated in figures and tables.

After 2 minutes preillumination of the sample in a medium without $MgCl_2$ and $NaH^{14}CO_3$, these compounds were added to start $^{14}CO_2$ fixation (see results on light activation and Mg^{2+}). For kinetic studies on metabolite compartmentation protoplasts were incubated in a total volume of 1 ml. At intervals 90 μ l was transferred to a centrifugation tube with a pipette, and the protoplasts were sedimented by a short acceleration in a Beckman Microfuge B. From the supernatant 70 μ l was added to 35 μ l of 0.6% Catran (1-methyl-2-phenyl-ethylhydrazine-HCl) in 1.0 M HCl, and subsequently 50 μ l 0.2% Catran in 0.33 M HCl was added to the drained pellet. At intermediate in-

tervals 90 μ l was withdrawn with a Hamilton syringe to produce a protoplast extract (about 80% intact chloroplasts), and the chloroplasts were sedimented by a 5 s centrifugation. Supernatants and pellets were treated as above.

Soluble products of $^{14}\text{CO}_2$ fixation, including Catran derivatives of keto acids, were separated by thin layer electrophoresis. Radioactive products were located by autoradiography, removed from the plates, and the radioactivity in each spot was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Light activation and Mg^{2+}

Recent investigations by Huber and Maury (8) show that Mg^{2+} inhibits CO_2 fixation and some related reactions in isolated chloroplasts from both C_3 and C_4 plants, if the Mg^{2+} is present in the medium before the onset of light.

We found inhibitory effects of Mg^{2+} also with our material (Fig. 1 a,b: B and D). Both OAc and PGA reduction were strongly inhibited already at a Mg^{2+} concentration of 1 mM, if the Mg^{2+} was added in the dark. Addition of 2 mM EDTA in excess, in the light, restored OAc reduction entirely, whereas PGA reduction was only partly restored. If the Mg^{2+} instead was added after 2 min preillumination of the material there was no inhibition of OAc reduction, and much less inhibition of PGA reduction (Fig. 1 a,b: A and C).

Since the PEP carboxylase is a Mg^{2+} dependent enzyme, $^{14}\text{CO}_2$ fixation with C_4 mesophyll protoplasts can not be run without Mg^{2+} . As a consequence of the experiments above, a preillumination period of 2 min in the absence of

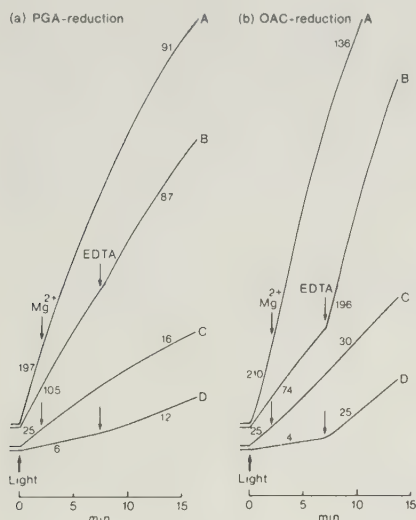


Fig. 1. a) PGA-reduction, and b) OAc-reduction with intact protoplasts (C,D), and protoplast extracts (A,B). The figures at the O_2 traces give the rates as $\mu\text{mol substrate reduced/mg Chl} \cdot \text{h}$. In A and C the medium contained 2 mM EDTA and no Mg^{2+} at the onset of light; and 3 mM Mg^{2+} was added after 2 min as indicated. In B and D the medium contained 1 mM Mg^{2+} added in the dark; and 3 mM EDTA was added after 7 min in the light. Substrate concentrations were: PGA, 2 mM; OAc, 4 mM.

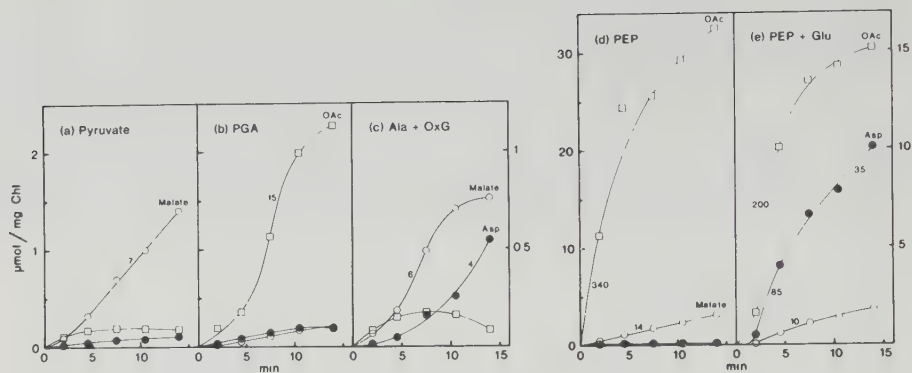


Fig. 2. Export of $^{14}\text{CO}_2$ fixation products from intact mesophyll protoplasts of the C_4 plant *D. sanguinalis*. The protoplasts were supplied with $\text{NaH}^{14}\text{CO}_3$ and other substrates as indicated (a - e). Concentrations were: pyruvate, 10 mM; PGA, 5 mM; Ala, 10 mM; OxG, 2 mM; PEP, 10 mM; Glu, 2 mM. At intervals 90 μl samples were withdrawn from the 1 ml reaction mixture. The protoplasts were pelleted, and the supernatants were analysed for export products. Oxaloacetate ($\square$ — $\square$), malate ($\circ$ — $\circ$), and aspartate ($\bullet$ — $\bullet$) together contained more than 95% of the ^{14}C recovered in the supernatant. The figures at the curves give the export rates as $\mu\text{mol/mg Chl} \cdot \text{h}$. Abbreviations: Ala, alanine; Asp, aspartate; OAc, oxaloacetate; OxG, 2-oxoglutarate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate.

Mg^{2+} (and $\text{NaH}^{14}\text{CO}_3$) and with 2 mM EDTA was used in the $^{14}\text{CO}_2$ fixation experiments. This procedure allowed maximal activities of all enzymes involved.

Export kinetics

Export kinetics of $^{14}\text{CO}_2$ fixation products from intact protoplasts supplied with different substrates are shown in Fig. 2 (a - e). With all substrates more than 80% of the ^{14}C incorporated was found outside the protoplasts after 7.5 min. Pyruvate (a), PGA (b), and Ala + 2-oxoglutarate (c) induced rates of $^{14}\text{CO}_2$ fixation and of export that were in the order of 10 $\mu\text{mol/mg Chl} \cdot \text{h}$. Only with PEP as substrate (d,e) rates were obtained that are comparable to those of intact leaves, i.e. 300 - 400 $\mu\text{mol/mg Chl} \cdot \text{h}$. The rate of malate formation and export was low ($\leq 14 \mu\text{mol/mg Chl} \cdot \text{h}$) with all substrates, compared to the rates expected from the role of malate as a main transport compound in NADP-malic enzyme species. Instead, high rates of OAc export were obtained (d,e). Addition of amino nitrogen stimulated formation and export of Asp (c,e) which otherwise was a minor product. PGA (b) induced OAc export and suppressed malate formation, whereas malate was the main product with pyruvate (a) and Ala + OxG (c). This indicates that PGA and OAc reduction competed for available NADPH. As a whole the composition of export products was determined by the substrate(s) used.

TABLE I

Products formed from (1- 14 C)PGA by mesophyll protoplasts during 15 min in the light. Incubation procedure as for 14 CO₂ fixation, except that cold NaHCO₃ was used. PGA concentration: 2 mM. Purity of 14 C-PGA: > 99%. About half of the PGA added was consumed during the experiment. Abbreviations: DHAP, dihydroxyacetonephosphate; HMP, hexose monophosphates; SP, sugar phosphates.

Product (nmol/mg Chl)	Control		+ 2 mM Pyruvate	
	Sup	Pellet	Sup	Pellet
DHAP	3200	76	1600	35
HMP	220	6	190	4
Other SP	620	9	590	7
PGA reduction	(4040)	(91)	(2380)	(46)
OAc	1600	41	1400	30
Malate	760	26	1200	33
Asp	310	20	120	7
CO ₂ fixation	(2670)	(87)	(2720)	(70)
PEP	160	5	180	4
Ala	160	-	7	2
Glu	130	-	100	-
Others	390	3	330	2
Total (%)	97.6	2.4	97.9	2.1

Fate of 14 C-PGA

The competition of PGA and OAc reduction for available NADPH was further investigated with 14 C-PGA in the presence or absence of pyruvate (Table I). In the presence of pyruvate less dihydroxyacetone phosphate (DHAP) was formed from PGA. Thus, pyruvate (or rather OAc formed from pyruvate) suppressed PGA reduction as expected, whereas the amount of PGA used as substrate for CO₂ fixation was not affected by pyruvate. Most of the DHAP formed was exported from the protoplasts, in agreement with the suggested flow of DHAP back to the bundle sheath cells (1).

Pool size kinetics

Kinetic studies on the compartmentation of C₄ acids were performed with the substrates used in Fig. 2. Results of two such experiments are shown in Fig. 3. The interpretation of the experiment with Ala + OxG as substrate is relatively straightforward: The protoplast pool is for all products larger than the chloroplast pool, and the values for Sup + Cyt are larger than those for Sup alone. However, some discrepancies are found: The difference between Sup + Cyt and Sup does not agree with the corresponding difference between the protoplast and the chloroplast pellet. This is especially evident for OAc. With pyruvate as substrate more discrepancies are found: The

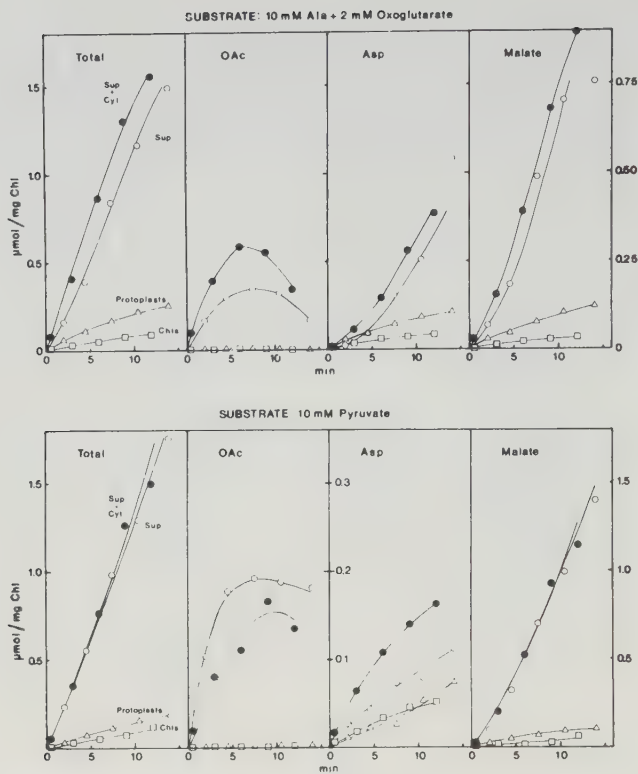


Fig. 3. Compartmentation of total products, OAc, Asp and malate during $^{14}\text{CO}_2$ fixation with intact mesophyll protoplasts. Substrates as indicated. At intervals, intact protoplasts were pelleted to produce a protoplast pellet ($\triangle-\triangle$) and a supernatant (Sup, $\circ-\circ$). At intermediate intervals, chloroplasts were pelleted from freshly broken protoplasts to produce a chloroplast pellet (Chls, $\square-\square$) and a supernatant containing both export products and the compounds of the cytoplasm (Sup + Cyt, $\bullet-\bullet$). Abbreviations: see Fig. 2.

chloroplast pool of Asp is initially larger than the protoplast pool, and the Sup values for OAc are larger than the Sup + Cyt values. By also withdrawing samples for analysis of total contents (protoplasts + Sup) during the experiments (data not shown) these discrepancies could partly be explained.

For all products, the total analysis and the sum of the protoplast pellet and Sup agreed. This was not the case when the total analysis was compared with the sum of the chloroplast pellet and Sup + Cyt. In this case only the values for malate agreed. It seems that when the protoplasts are broken by passage through the Hamilton syringe, amino groups are made available for the aminotransferase reaction. Thus, OAc was taken up from Sup + Cyt and converted to Asp inside the chloroplasts. This was especially the case if

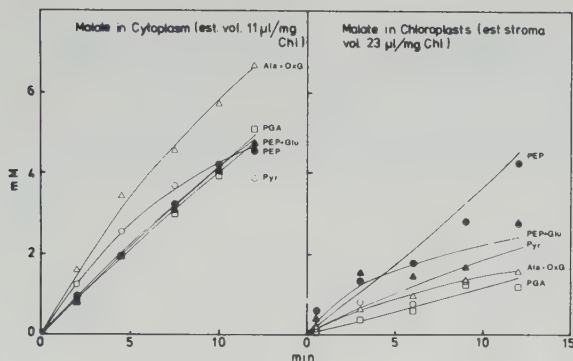


Fig. 4. Kinetics of the cytoplasm and chloroplast pools of ^{14}C -malate in mesophyll protoplasts supplied with different substrates (Pyr, $\circ$ — $\circ$; PGA, $\square$ — $\square$; Ala + OxG, $\triangle$ — $\triangle$; PEP, $\bullet$ — $\bullet$; PEP + Glu, $\blacktriangle$ — $\blacktriangle$). Pool sizes are calculated from experiments such as those in Fig. 3. The cytoplasm pool was taken as the difference between the protoplast and the chloroplast pellet. Volume estimations: see Fig. 5.

amino groups were not made available throughout the experiment and the chloroplast pool thereby far from saturated. Therefore only total protoplast pools are calculated for OAc and Asp in Fig. 5, except for the experiment with Ala + OxG. The amino nitrogen released upon breakage of the protoplasts may be derived from the tonoplast, since its contents are relatively isolated from the other compartments in the intact protoplast.

Since the values obtained for malate seem to be reliable, as judged from the agreement of total analysis with the sums of protoplasts + Sup, and Chls + (Sup + Cyt), respectively, the chloroplast and cytoplasm pools of malate were calculated for experiments with different substrates (Fig. 4). Neither the cytoplasm, nor the chloroplast pools were saturated during the experiment. The cytoplasm pools were strikingly similar independent of the substrate supplied, whereas the chloroplast pools showed larger differences.

Internal pool sizes

From the compartmentation experiments we have calculated the internal pools of the three C_4 acids after 10.5 min of $^{14}\text{CO}_2$ fixation (Fig. 5). Compartment volumes were estimated from electronmicrographs (see legend). The cytoplasm pool of malate was almost independent of substrate supplied, of $^{14}\text{CO}_2$ fixation rate, and of export rate of malate (compare Fig. 2). The chloroplast pool, on the other hand, was more proportional to the rate of malate export (and thereby to OAc reduction). The intraprotoplast pool of OAc was low, except with PEP or PEP + Glu as substrates. The chloroplast and cytoplasm pools of Asp were in the same range as those of malate with

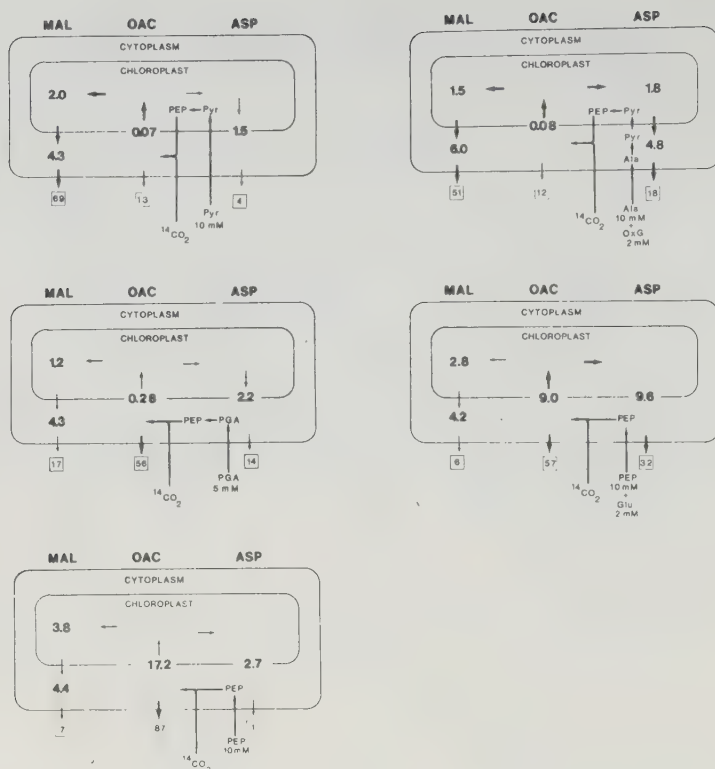


Fig. 5. Estimated pools (mM) of the three C₄ acids after 10.5 min of ¹⁴CO₂ fixation with intact protoplasts. Figures within □ are percent of total. Arrows indicate the flow of metabolites. Values placed on the chloroplast border are average protoplast concentrations (Chls + cytoplasm, for reasons, see Pool size kinetics). Substrates as indicated. Absolute values for the different pools were obtained from time-course experiments (Fig. 3). Cytoplasm values were taken as the difference between protoplast and chloroplast pools. Compartment volumes were estimated as follows: Protoplast diameter (light microscopy) and chlorophyll per protoplast was determined. The protoplast was approximated with a sphere - the vacuole as an inner sphere and chloroplasts and cytoplasm as spherical shells outside the vacuole. From electronmicrographs of intact cells, the cross section area of these volumes were determined and the corresponding radii and compartment volumes were calculated. Data obtained: Diameter, 17.4 μm; Chl/cell, 2.42 · 10⁻⁵ μg; Volume, 114 μl/mg Chl; Relative compartment volumes: vacuole 50%, chloroplasts 40% (stroma 20%), cytoplasm 10%. We have assumed that the vacuole is metabolically nonactive and that the mitochondrial volume is negligible (<1%). The volume of the nucleus is included in the cytoplasm.

Ala + OxG as substrate. The average chloroplast + cytoplasm value for this experiment is 2.8 mM, and thus similar to the intraprotoplast pool sizes obtained with pyruvate, PGA and PEP. Only with high ¹⁴CO₂ fixation rate and amino nitrogen present (PEP + Glu) the Asp pool reached higher values.

The fact that the malate and Asp pools were relatively independent of export rates indicates a regulated transport of these compounds across the plasma membrane. By contrast, the size of the OAc pool was proportional to the export rate, indicating that OAc passed the membrane by free diffusion.

GENERAL DISCUSSION

In C_4 plants of the NADP-malic enzyme type, such as *D. sanguinalis*, mesophyll cells are supposed to export mainly malate, with pyruvate obtained from the bundle sheath cells as main substrate. One would therefore expect that intact mesophyll protoplasts should be able to fix $^{14}CO_2$ and export malate at rates comparable to the CO_2 fixation rates of the intact leaves ($300 - 400 \mu\text{mol/mg Chl} \cdot \text{h}$) when supplied with pyruvate and $\text{NaH}^{14}CO_3$.

As seen in Fig. 2 this was not the case. All substrates except PEP induced low rates of $^{14}CO_2$ fixation and export. Malate was the main product with some substrates (e.g. pyruvate), but the rate of export was always low ($\leq 14 \mu\text{mol/mg Chl} \cdot \text{h}$). When high $^{14}CO_2$ fixation rates were obtained, the main product was OAc, or OAc and Asp.

Malate and Asp both inhibit the PEP carboxylase in vitro (9). The low $^{14}CO_2$ fixation rates can, however, not be explained by high cytoplasmic concentrations of these compounds, since the pool sizes were relatively equal and independent of the $^{14}CO_2$ fixation rates (compare Figs. 5 and 2). We believe that the concentration of PEP in the cytoplasm limited $^{14}CO_2$ fixation, since only PEP itself gave high rates.

Chloroplasts obtained from protoplasts certainly have the ability to reduce OAc to malate at high rates (Fig. 1b: A), whereas intact protoplasts reduce OAc at much lower rates (Fig. 1b: C). This can not be due to low rates of OAc uptake since OAc readily passes the plasma membrane as seen from the export curves (Fig. 2). Moreover, a high intraprotoplast concentration of OAc (17 mM, Fig. 5) does not automatically lead to a high rate of malate formation. The low rate of malate production by intact protoplasts must be explained by some other factor, e.g. limitations in noncyclic electron transport or in exchange of dicarboxylic acids across the chloroplast envelope.

With the isolation of protoplasts the plasmodesmata are lost. Excretion of products from intact protoplasts should therefore be due to diffusion and/or transport across the plasma membrane. Our results show that the plasma membrane of the mesophyll protoplasts permits high rates of OAc export ($340 \mu\text{mol/mg Chl} \cdot \text{h}$) and also relatively high rates of Asp export ($85 \mu\text{mol/mg Chl} \cdot \text{h}$), while the low rates of malate export rather seem to be

due to low rates of malate synthesis than to properties of the plasma membrane restricting the transport.

The high rates of OAc export and low rates of malate export which we have obtained with mesophyll protoplasts are therefore probably an artefact, and due to the suppressed rate of OAc reduction. The situation in situ can be expected to be the opposite - that is high rates of malate export and low rates of OAc export. Our results suggest that this export is regulated and occurs via the plasma membrane - cell wall path, in contrast to the generally held view of free diffusion through plasmodesmata (1,4).

The present concept of plasmodesmata (4) means that the plasma membrane of a plant cell is continuous with the plasma membrane of adjacent cells via channels through the cell wall, thus allowing free diffusion of small molecules in between the cells. We would rather believe that the plasmodesmata are channels through the cell wall connecting patches of specific transport proteins in the plasma membranes of adjacent cells. If that is the case transport via plasmodesmata would be very similar to what presently is known as transport via the plasma membrane - cell wall path (4).

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Highly purified intact chloroplasts from mesophyll protoplasts of the C_4 plant *Digitaria sanguinalis*. Inhibition of phosphoglycerate reduction by orthophosphate and by phosphoenolpyruvate

Mats Hallberg and Christer Larsson

Hallberg, M. and Larsson, C. 1983. Highly purified intact chloroplasts from mesophyll protoplasts of the C_4 plant *Digitaria sanguinalis*. Inhibition of phosphoglycerate reduction by orthophosphate and by phosphoenolpyruvate. — *Physiol. Plant.* 57: 330–338.

Purified mesophyll protoplasts from the C_4 plant *Digitaria sanguinalis* were used to prepare intact mesophyll chloroplasts with low cytoplasmic contamination. The procedure involved breakage of protoplasts, differential centrifugation, partition in a dextran-polyethylene glycol two-phase system, and Percoll density gradient centrifugation. The final chloroplast preparation contained about 80% intact chloroplasts with a phosphoenolpyruvate carboxylase contamination of 0.2–1% of the original protoplast activity, corresponding to 1–6 $\mu\text{mol } ^{14}\text{CO}_2 \text{ fixed/mg Chl} \cdot \text{h}$. The purified chloroplasts showed substrate-dependent oxygen evolution in the range of 40–150 μmol substrate reduced/mg Chl $\cdot \text{h}$, with phosphoglycerate or oxaloacetate as substrate. Both reactions were stimulated 1.5 fold by pyruvate and further by addition of the other substrate. These measurements indicated that phosphoglycerate reduction was limited by substrate transport across the chloroplast envelope. Without added substrate, the chloroplasts consumed oxygen via pseudo-cyclic electron transport in the light. Also this reaction was stimulated by pyruvate. Phosphoglycerate-dependent oxygen evolution was inhibited by P_i and by phosphoenolpyruvate to about the same extent with purified chloroplasts, but only by P_i with protoplast extracts. This suggests that phosphoglycerate, P_i and phosphoenolpyruvate share a common carrier, similar to the P_i -translocator in C_3 chloroplasts, and that the lack of inhibition obtained with phosphoenolpyruvate and unpurified chloroplasts is artefactual, possibly due to oxaloacetate formation from added phosphoenolpyruvate and concomitant stimulation of oxygen evolution by oxaloacetate reduction. Furthermore, the results suggest that phosphoenolpyruvate is transported with a K_m similar to that of P_i in C_4 mesophyll chloroplasts.

Additional key words — Percoll gradient centrifugation, phase partition, P_i -translocator, transport.

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Introduction

In C_4 plants of the NADP-malic enzyme group, such as *Digitaria sanguinalis* and *Zea mays*, the mesophyll chloroplasts are highly specialized organelles which carry out three of the major reactions of C_4 photosynthesis: conversion of pyruvate to PEP, reduction of OAc to malate, and reduction of PGA to DHAP. Pyruvate, originating in the bundle sheath cells, is converted

to PEP in the mesophyll chloroplasts, exported and carboxylated to OAc in the cytoplasm. OAc is taken up by the chloroplasts and reduced to malate, which is exported from the mesophyll cells to the bundle sheath cells, where it is split into CO_2 and pyruvate, and the CO_2 is refixed via the Calvin cycle. Part of the PGA formed in this second carboxylation is transported back to the mesophyll cells and reduced to DHAP in the

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chloroplasts. (For a review see Hatch and Osmond 1976).

All of these metabolic steps require transport of compounds across the chloroplast envelope. *Digitaria* mesophyll chloroplasts have, in uptake experiments with labelled compounds, been shown to have carriers for PEP/ P_i exchange and for pyruvate (Huber and Edwards 1977a, b). Maize mesophyll chloroplasts have in similar experiments been shown to transport P_i , PGA, DHAP and PEP on a common exchange-diffusion carrier (Day and Hatch 1981b) which is probably similar to the P_i -translocator of C_3 chloroplasts (Fliege et al. 1978). PGA-dependent O_2 evolution can be used to monitor effects of different compounds on the transport via the P_i -translocator. However, although PEP and P_i seem to have similar K_m 's for this translocator, which they share with PGA, it has not been possible to inhibit PGA-dependent O_2 evolution with PEP, but only with P_i (Edwards et al. 1979, Day et al. 1981). Thus, the results obtained in uptake experiments are not confirmed by the results obtained in inhibition studies on PGA-dependent O_2 evolution.

In the experiments above chloroplasts were used, which had been prepared by gentle breakage of isolated protoplasts and collection of the chloroplasts by a single centrifugation. We consider the reason for the conflicting results on the properties of the P_i -translocator in C_4 mesophyll chloroplasts to be the purity of the chloroplasts used.

In this paper we report the preparation and properties of intact, highly purified mesophyll chloroplasts from *Digitaria sanguinalis*. With these chloroplasts PGA-dependent O_2 evolution is inhibited both by P_i and by PEP. Furthermore, the results of the inhibition studies support earlier results obtained with other methods (Huber and Edwards 1977b, Day and Hatch 1981b), which indicate that P_i and PEP have similar K_m 's for the P_i -translocator in C_4 mesophyll chloroplasts, in contrast to the situation in C_3 chloroplasts, where PEP is a poor substrate for the translocator.

Abbreviations – BSA, bovine serum albumin (fatty acid free); Chl, chlorophyll; DTT, dithiothreitol; Hepes, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; OAc, oxaloacetate; PEG, polyethylene glycol; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; Pyr, pyruvate; R-5-P, ribose-5-phosphate; DHAP, dihydroxyacetone phosphate.

Materials and methods

Chemicals

Dextran T 500 and Percoll were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Polyethylene glycol 4000 (Carbowax 4000, recently renamed as Carbowax 3350) was from Union Carbide, New York.

$NaH^{14}CO_3$ (60 Ci/mol; 2.22 TBq/mol) was from The Radiochemical Center, Amersham.

Plant material

Leaves from 16–18 day-old plants of crabgrass [*Digitaria sanguinalis* (L.) Scop.] were used. Plants were cultured in standard potting soil in a growth chamber with a day length of 16 h and a day/night temperature of 30/25°C. Dysprosium lamps (Osram HQIL, 400 W) gave a quantum flux density of 230–400 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 400–700 nm.

Isolation of mesophyll protoplasts

Two different procedures were used for preparation of leaf segments and enzymatic digestion.

A: Leaves, two at a time, were cut into 0.7 mm wide segments with a mechanical leaf cutter (Huber and Edwards 1975d), and the segments were collected in 0.5 M sorbitol, 1 mM $MgCl_2$, 1 mM KH_2PO_4 , 0.1% BSA, adjusted to pH 6.0 with KOH. Eight grams of washed segments were suspended and partially vacuum infiltrated in 400 ml of: 2% Driselase (Kyowa Hakko Co. Ltd., Tokyo, Japan), 0.5 M sorbitol, 1 mM $MgCl_2$, 1 mM KH_2PO_4 , 0.1% BSA, adjusted to pH 5.5 with KOH. The enzymatic digestion was performed at 30°C on a waterbath with continuous shaking during the first half hour, and gentle swirling of the solution every half hour during the following 1.5 h.

B: About twenty leaves at a time were cut by hand with a razor blade into 1–2 mm wide segments. Washed segments (8 g) were incubated with 50 ml of the enzymatic digestion medium in a 15 cm Petri dish, covered with a glass lid without shaking for 2 h. During the incubation the segments were illuminated with incandescent light through a heat filter, giving a quantum flux density of 100–150 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 400–700 nm.

Protoplasts were released from the digested leaf segments and purified by partition in an aqueous polymer two-phase system as previously described (Hallberg and Larsson 1981).

Method B gave a shorter preparation time and protoplasts with higher activities. However, the yield of purified protoplasts was only half of that obtained with method A. With method A the enzyme solution could be used a second time after centrifugation at 7000 g for 20 min.

Protoplast extracts

Protoplast extracts were prepared immediately before use by passing protoplasts through a 100 μl Hamilton syringe (Gutierrez et al. 1975).

Preparation of intact mesophyll chloroplasts

Protoplasts, 5 ml with 100–400 µg Chl/ml, suspended in the upper phase from the final purification step were used for preparing chloroplasts.

The protoplast suspension was diluted with 5 ml of medium I: 0.5 M sorbitol, 50 mM Hepes-KOH, pH 7.6, 0.5 mM KH_2PO_4 , 0.1% BSA, to which was added 5 mM ascorbate and 4 mM cysteine just before use. Protoplasts were pelleted at 200 g for 2 min and the pellet was suspended in 10 ml of medium II, composed as medium I except that the sorbitol concentration was 0.4 M and with ascorbate and cysteine added as above. The protoplasts were broken by passing the suspension six times through a 20 µm nylon net mounted on the cut end of a 20 ml disposable syringe (Rathnam and Edwards 1976). The released chloroplasts were pelleted at 3600 rpm (1600 g) for 5 s in a Sorvall SS-34 rotor. The chloroplasts were suspended in 10 ml of medium II and pelleted at 1500 rpm (270 g) for 40 s. The resulting pellet of washed chloroplasts was resuspended to a total volume of 1.5 ml in 0.4 M sorbitol, 5 mM potassium phosphate, pH 7.8 and 0.1% BSA.

Purification of chloroplasts

Phase partition

To remove unbroken protoplasts and multiorganelle complexes (Larsson and Albertsson 1974), the chloroplasts were partitioned in an aqueous polymer two-phase system containing: 6.2% (w/w) Dextran T 500, 6.2% (w/w) PEG 4000, 0.40 M sorbitol, 5 mM potassium phosphate, pH 7.8 and 0.1% BSA. A 6.0 g phase mixture was made in a Sorvall SS-34 centrifugal tube by mixing: 2.325 g of 20% (w/w) Dextran T 500, 1.162 g of 40% (w/w) PEG 4000, 1.200 g of a sorbitol solution containing 2 mol/kg, 0.150 g of 0.2 M potassium phosphate, pH 7.8, 0.006 g BSA, and finally 1.157 g of distilled water. After adding 1.5 ml of the chloroplast suspension, containing 500–700 µg of chlorophyll, to this phase mixture, a 7.5 g phase system with the desired final composition was obtained. The tube was swirled by hand to mix the system, and the phases were allowed to settle for 10–15 min at unit gravity. The upper phase was withdrawn with a Pasteur pipette and discarded. The lower phase together with the interface was repartitioned with 4.5 g of fresh upper phase from a large phase system (Larsson and Andersson 1979, Albertsson et al. 1981) of the same composition. The second upper phase was removed and discarded as before. The resulting lower phase and interface, containing intact and broken chloroplasts, was diluted four times with medium II to a final volume of 8–10 ml to lower the viscosity of the suspension.

Percoll gradient centrifugation

Intact chloroplasts were further purified from broken ones by centrifugation through a Percoll gradient (Price

et al. 1979, Mills and Joy 1980). Either of the following two gradients were used:

(1) A discontinuous gradient consisting of 2 ml 63% (v/v) Percoll and 4 ml 40% (v/v) Percoll in 5 times diluted medium II containing 0.4 M sorbitol. The diluted chloroplast suspension was layered on top of the gradient in a Sorvall SS-34 centrifugal tube and the gradient was spun at 1800 rpm (500 g max) for 5 min in a swinging bucket centrifuge (MSE).

Intact chloroplasts banded against the 63% layer. The layers above were removed with a Pasteur pipette and the chloroplast band collected. The Percoll-containing chloroplast suspension was diluted with half its weight of a medium to give the composition of medium II, and was stored on ice.

(2) The modified gradient contained only 4 ml of the 40% (v/v) Percoll solution used above. The procedure was as for (1) except that the intact chloroplasts formed a loose pellet under the 40% layer.

All steps during isolation and purification of chloroplasts were performed in the cold room at 2–4°C.

PEP carboxylase assays

In the spectrophotometric assay the medium contained 50 mM Hepes-KOH, pH 7.6, 5 mM NaHCO_3 , 10 units of malate dehydrogenase, 0.15 mM NADH, and a sample corresponding to 0.2–6 µg Chl depending on activity, in a total volume of 1 ml. The oxidation of NADH was followed at 340 nm with an Aminco DW2 spectrophotometer. The baseline was recorded and after temperature equilibration (30°C) the reaction was started by addition of 5 mM PEP.

In the assay using radioactive bicarbonate the samples were preactivated for 90 min at 22°C essentially according to Hatch and Oliver (1979) in 50 mM Hepes-KOH, pH 7.6, 5 mM MgCl_2 , 5 mM DTT and 0.5% BSA. Assays were performed in test tubes on a water-bath at 30°C in a total volume of 100 µl. The reaction was started by adding 5 mM PEP and 3.4 mM $\text{NaH}^{14}\text{CO}_3$ and was terminated after 6 min by the addition of 50 µl 1 M HCl. The incorporation of radioactive carbon into non-volatile products was determined by liquid scintillation counting of 20 µl aliquots spotted on filter papers.

$^{14}\text{CO}_2$ fixation was run in medium II with 1.3 µg Chl in a total volume of 100 µl. The reaction was started by adding 3.4 mM $\text{NaH}^{14}\text{CO}_3$ and turning on the light (slide projector). The assay was otherwise performed and terminated as above for PEP carboxylase.

Oxygen conversion

The intactness of the chloroplasts was calculated from the ferricyanide-dependent oxygen evolution by intact

and osmotically shocked chloroplasts (5 mM MgCl₂ in shocking medium) according to Heber and Santarius (1970). Catalase was included in the medium (5500 units/ml) to reduce oxygen consumption due to pseudocyclic electron transport (see Tab. 3).

Substrate-dependent oxygen conversion was measured at 30°C in a total volume of 1 ml with a Clark-type oxygen electrode (Hansatech Ltd., King's Lynn, Norfolk). The assays were performed in medium II also containing 2 mM EDTA, 5500 units of catalase, and chloroplasts corresponding to 2–13 µg of chlorophyll. Substrates and other additions were as indicated in Tab. 3 and Fig. 3. Light (2500 µmol·m⁻²·s⁻¹ at 400 to 700 nm) was provided by a slide projector. Chlorophyll was determined according to Arnon (1949).

Electron microscopy was essentially as described elsewhere (Larsson et al. 1971).

Results

The final preparation of intact mesophyll chloroplasts could be obtained within 1.5 h after the protoplasts were broken, and the yield was 15–20% of the chlorophyll originally found in the protoplasts (Tab. 1). The chloroplasts were examined in the phase contrast microscope (Fig. 1C) and judging from their appearance (intact chloroplasts brighter than broken ones) they were about 80% intact. This result was confirmed by the ferricyanide test which gave values between 80 and 97%.

An electron micrograph of the purified chloroplasts (Fig. 2) shows that they are intact and without any adsorbed layer of cytoplasm. Invaginations of the inner envelope membrane, the so-called peripheral reticulum (Laetsch 1974), can be observed.

Contamination by cytoplasm was monitored by the levels of PEP carboxylase (Tab. 1). Once washed chloroplasts still contained about 20% of the activity found in protoplasts, but this contamination was efficiently reduced by phase partition. In this step the remaining intact protoplasts were removed with the upper phase (Fig. 1A), while intact chloroplasts were left at the interface and in the lower phase (Fig. 1B). Percoll gradient centrifugation was used to increase the intactness of the isolated chloroplasts (Fig. 1C) and served also to reduce the contamination by PEP carboxylase. The residual PEP carboxylase activity was always in the range of 1–6 µmol ¹⁴CO₂ fixed/mg Chl·h. The possibility that these low rates were due to inactivation of PEE carboxylase by the low temperature used during chloroplast preparation was investigated by assaying the enzyme after preactivation of the samples (Tab. 1). There was little or no stimulation of the activities by this treatment.

Tab. 1. Yield of chlorophyll and levels of PEP carboxylase at different stages in the chloroplast preparation procedure. Values are given for two preparations (I, II) starting from 8.0 and 9.9 g of leaf segments, respectively. The first preparation was from protoplasts prepared according to method A, and the two step Percoll gradient was used. The second was from protoplasts prepared according to method B, and a single layer of Percoll was used to purify the chloroplasts. In preparation I, PEP carboxylase activities were measured with the spectrophotometric assay (see Materials and methods). With preparation II both the spectrophotometric and the radioactive assay were used. The latter assay included a preactivation of the enzyme (Hatch and Oliver, 1978). Activities are expressed as µmol NADH oxidized/mg Chl·h and (within brackets) as µmol ¹⁴CO₂ fixed/mg Chl·h.

Fraction	Preparation	Total Chl (µg)	PEP carboxylase (specific activity)
Purified protoplasts	I	1188	625
	II	1025	1136 (1135)
Chloroplasts pelleted twice	I	904	96.4
	II	715	199 (285)
Phase partitioned chloroplasts	I	323	14.6
	II	657	4.5 (6.6)
Chloroplasts from Percoll gradient	I	206	6.0
	II	198	3.3 (2.1)

Contamination by bundle sheath chloroplasts was measured as the stimulation of ¹⁴CO₂ fixation by R-5-P (Tab. 2). No such stimulation was found; instead PEP increased ¹⁴CO₂ fixation, confirming that the remaining CO₂ fixation capacity was due to PEP carboxylase rather than ribulose-1,5-biphosphate carboxylase. The PEP carboxylase activity with isolated intact chloroplasts, measured as ¹⁴CO₂ fixation in the light, was generally lower than the activity determined spectrophotometrically as NADH oxidation (compare Tabs 1 and 2).

Substrate-dependent oxygen conversion

The rate of PGA-dependent oxygen evolution by isolated chloroplasts varied between values corresponding to 55–140 µmol PGA reduced/mg Chl·h (mean 92, n = 13) in different preparations. There was usually a lag of 2 to 5 min before the maximal activity was reached. The addition of 2 mM pyruvate stimulated the activity 1.4 fold (Tab. 3), and O₂ evolution increased further when OAc was added. The rate of malate formation, measured as OAc-dependent oxygen evolution, was between 26 and 115 µmol OAc reduced/mg Chl·h (means 61, n = 9). Pyruvate stimulated also this reaction about 1.5 fold (2 mM). A subsequent addition of PGA increased O₂ evolution further (Tab. 3).

When chloroplasts were illuminated without substrate they showed oxygen uptake (Tab. 3). This pseudocyclic electron transport (Huber and Edwards 1975c) was stimulated by pyruvate. The addition of

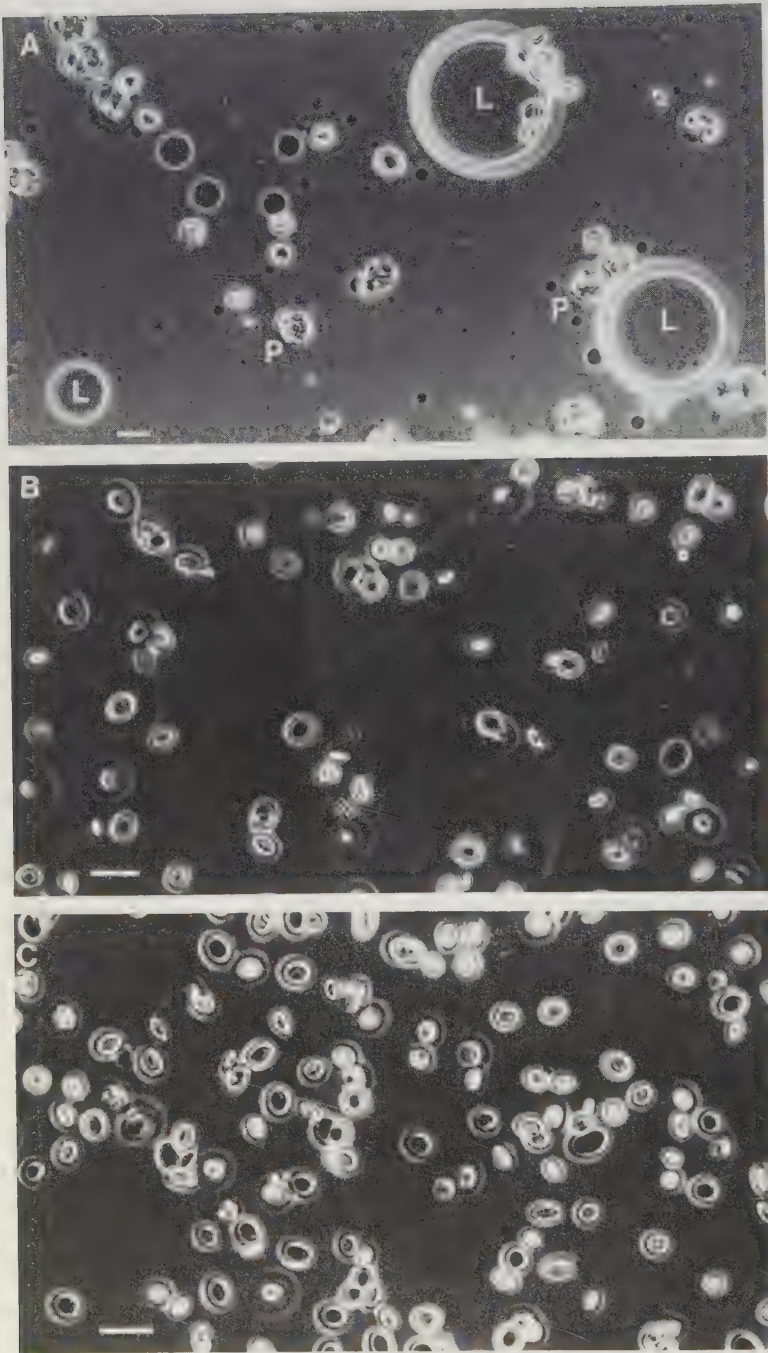


Fig. 1. Phase contrast light micrographs of different fractions from the preparation procedure of *Digitaria* mesophyll chloroplasts. A, material removed with the upper phase in the phase partitioning of chloroplasts. Note remaining intact protoplasts (P) and droplets of the lower phase (L). B, material collected in the lower phase and at the interface – mainly intact chloroplasts. C, final chloroplast fraction obtained after Percoll gradient centrifugation – about 80% intact chloroplasts. Bars represent 20 μ m.

Fig. 2. Electron micrograph of mesophyll chloroplasts after the last purification step (compare Fig. 1C). Note the peripheral reticulum (PR).



10–100 μM azide, a catalase inhibitor, increased the rate slightly, indicating that a low amount of catalase may be present in the chloroplast preparations.

Inhibition of PGA reduction

The effect of increasing P_i or PEP concentrations in the medium during PGA reduction was studied. Both P_i and PEP inhibited PGA reduction and to about the same extent with isolated chloroplasts (Fig. 3A).

Tab. 2. Light-dependent $^{14}\text{CO}_2$ fixation, expressed as $\mu\text{mol } ^{14}\text{CO}_2 \text{ fixed/mg Chl} \cdot \text{h}$, by isolated mesophyll chloroplasts. Values are from the same two experiments as in Tab. 1.

Substrate	Preparation	
	I	II
None	0.512	0.241
5 mM PEP	1.67	1.71
5 mM R-5-P	0.395	0.239

Tab. 3. Light-dependent O_2 conversion with purified mesophyll chloroplasts. The reactions were run in 1 ml of 0.4 M sorbitol, 50 mM Hepes-KOH, pH 7.6, 0.5 mM KH_2PO_4 , 0.1% BSA (medium II) with 2 mM EDTA, 5500 units of catalase (not in oxygen consumption measurements) and chloroplasts corresponding to 8–10 μg of chlorophyll (with PGA and OAc reduction) or 16 μg (without initial substrate). Initial substrates were present before the light was turned on. Other substrates were consecutively added in the light after linear reactions were obtained; 2 mM Pyr for PGA- and OAc-reduction and 4 mM Pyr without initial substrate. Sodium azide was 10 μM in the first measurement and 100 μM in the second. Values are from three different preparations (not the same as in Tabs 1 and 2) with 80, 97 and 93% intact chloroplasts, respectively, as measured by the ferricyanide method. PGA and OAc reduction are given as μmol substrate reduced/mg Chl $\cdot$ h. Values within brackets are percentage of initial activity $\pm$ SD for the three experiments. Values for the oxygen consumption are given as $\mu\text{atoms oxygen/mg Chl} \cdot \text{h}$ and as percentage of initial (within brackets).

Initial substrate	Preparation	Initial rate	Consecutive additions			
			Pyruvate	2 mM OAc	4 mM PGA	Sodium azide
None	I	-16 (100)	-32 (200)	—	—	-38 (238)
	III	-12 (100)	-20 (166)	—	—	-30 (250)
4 mM PGA	I	137	207	236	—	—
	II	76 (100)	100 (140 $\pm$ 10)	115 (163 $\pm$ 10)	—	—
	III	55	75	90	—	—
2 mM OAc	I	74	103	—	177	—
	II	26 (100)	37 (147 $\pm$ 8)	—	53 (224 $\pm$ 16)	—
	III	42	66	—	95	—

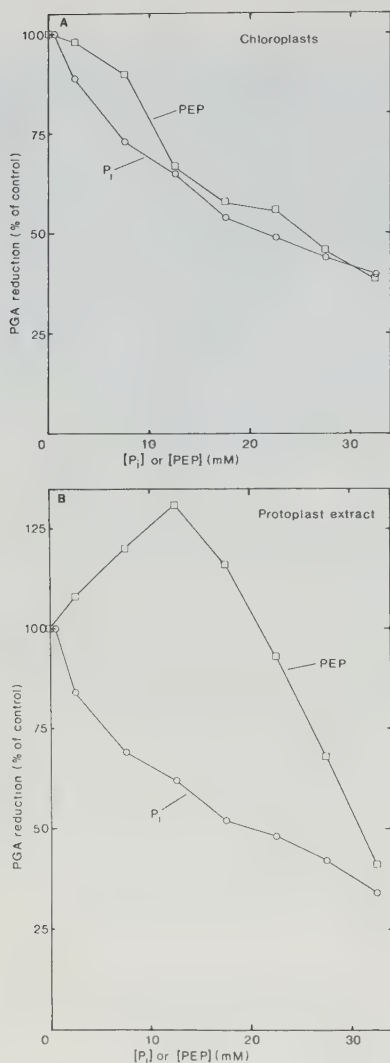


Fig. 3. Inhibition of PGA reduction by P_i and by PEP with purified mesophyll chloroplasts (A) and protoplast extract (B). Reactions were run in 1 ml of: 0.4 M sorbitol, 50 mM Hepes-KOH, pH 7.6, 0.5 mM KH_2PO_4 , 0.1% BSA (medium II) with 2 mM EDTA and 5500 units of catalase. PGA concentration was 2 mM and chlorophyll was 13.2 μ g for chloroplasts and 2.95 μ g for protoplast extract. Each measurement was started by recording the control rate in the above medium (including 0.5 mM P_i) and then the concentration of P_i (or PEP) was increased by consecutive additions of small volumes of concentrated solutions. In a separate measurement the time-dependent decrease in PGA reduction was recorded (30% in 25 min, which corresponded to the last addition point). Activities are given as percentage of the control rates and are corrected for the time-dependent decrease in PGA reduction. The control rates were for mesophyll chloroplasts 61 (P_i) and 63 (PEP) μ mol PGA reduced/mg Chl $\cdot$ h, and for protoplast extract 235 and 266 μ mol PGA reduced/mg Chl $\cdot$ h, respectively.

For comparison with unpurified chloroplasts the effect on PGA reduction of P_i and of PEP was measured also with protoplast extracts (Fig. 3B). The inhibition by P_i exhibited the same concentration dependence as with isolated chloroplasts. With PEP, however, oxygen evolution was stimulated at low or moderate PEP concentrations. Only at very high concentrations of PEP an inhibition of PGA-dependent oxygen evolution became evident.

Discussion

A method often used for preparing C_4 mesophyll chloroplasts involves the disruption of protoplasts by passing them through a 20 μ m nylon net and the collection of the chloroplasts by a single centrifugation at 250–400 g (Huber and Edwards 1975a, b, Edwards et al. 1978, Day et al. 1981). These authors reported chloroplasts with a residual PEP carboxylase activity of 2 to 5% of initial protoplast activity, corresponding to 50–100 μ mol $^{14}CO_2$ fixed/mg Chl $\cdot$ h. The intactness of once pelleted *Digitaria* mesophyll chloroplasts was 90–98% as measured by enzyme retention and ferricyanide reduction (Huber and Edwards 1977a, b). Similar values were obtained for *Panicum miliaceum* chloroplasts (Edwards et al. 1979). *Zea mays* mesophyll chloroplasts prepared in the same way were 80% intact (Day et al. 1981).

By combining differential centrifugation, phase partition and density gradient centrifugation we have obtained purified *Digitaria* mesophyll chloroplasts with a cytoplasm contamination corresponding to only 0.2–1% of the original PEP carboxylase activity, and with an intactness of 80–97%.

The high level of PEP carboxylase in once washed chloroplasts was due to the presence of unbroken protoplasts (Tab. 1 and Fig. 1A). *Digitaria* mesophyll protoplasts have been reported to have a diameter range of 10–28 μ m, with a mean of 21 μ m (Kanai and Edwards 1973) and we obtained values of 12–28 μ m, mean 17 μ m, SD = 3. In our hands the smallest protoplasts remained intact even after 6 passes through a 20 μ m nylon net. We also tried a 15 μ m nylon net but it was equally inefficient in breaking all of the protoplasts (data not shown). By subjecting the chloroplast preparation to phase partition, using a phase system similar to the one used for protoplast purification, but at 4°C, we could selectively remove the contaminating protoplasts. The effect of this purification step was drastic: the PEP carboxylase activity was reduced 7–45 fold (Tab. 1). Percoll density centrifugation was used as a final purification step, mainly to separate intact chloroplasts from broken ones. This was necessary, since intact and broken chloroplasts from *Digitaria* were not well separated by phase partition, in contrast to chloroplasts from spinach (Larsson et al. 1971).

The rates of PGA- and OAc-dependent oxygen evolution observed with the purified chloroplasts (Tab.

3 and Fig. 3A) are generally lower than those reported by others for NADP-malic enzyme type mesophyll chloroplasts (Huber and Edwards 1975a, protoplast extract; Day et al. 1981, once pelleted chloroplasts) and for NAD-malic enzyme type mesophyll chloroplasts (Edwards et al. 1979, once pelleted chloroplasts). Higher rates were obtained with freshly prepared protoplast extracts (Fig. 3B). The reason for this could be that the chloroplasts during the relatively lengthy preparation procedure have become depleted of P_i and dicarboxylic acids, that would exchange for external substrate during PGA and OAc reduction. Low endogenous stromal concentrations of P_i and malate have been observed with isolated maize mesophyll chloroplasts (Day and Hatch 1981a, b) as compared to spinach (Fliege et al. 1978, Lehner and Heldt 1978); and low saturated concentrations of P_i have been obtained in uptake experiments with *Digitaria* mesophyll chloroplasts (Huber and Edwards 1977a, b). Day and Hatch (1981b) suggested that either leakage from protoplasts during enzyme digestion of the tissue, a larger stromal volume, or inherent low metabolic concentrations in C_4 mesophyll chloroplasts was the reason for this difference compared to C_3 chloroplasts.

An alternative reason for the relatively low rates of PGA and OAc reduction obtained with the purified chloroplasts is that they may have lost nucleotides, since *Digitaria* mesophyll chloroplasts have been shown to transport ATP in exchange for internal ADP at high rates compared to spinach (Huber and Edwards 1976). Loss of nucleotides would lead to a low phosphorylation rate and thereby to a low rate of non-cyclic electron transport, which would limit both PGA and OAc reduction. The higher activities obtained with protoplast extracts, containing freshly liberated chloroplasts, suggest that leakage was the main reason for the lower activities with purified chloroplasts. The very large surface of the inner envelope membrane of C_4 mesophyll chloroplasts, caused by the peripheral reticulum (Laetsch 1974), may increase the loss of endogenous metabolites during isolation of these chloroplasts as compared to C_3 chloroplasts.

The stimulation of PGA reduction by pyruvate (Tab. 3) has been proposed to be due to uncoupling of non-cyclic electron transport by the enzyme pyruvate- P_i -dikinase, which catalyzes the formation of PEP from pyruvate and ATP (Kagawa and Hatch 1974, Day et al. 1981). If this were the case, the interpretation would be that initially PGA reduction was not limited by the supply of ATP. Subsequent addition of OAc stimulated oxygen evolution even more (Tab. 3); a stimulation was also seen if OAc was added before pyruvate (data not shown). This suggests that PGA reduction was not limited by NADPH either.

Thus, it seems that PGA reduction was limited by some other factor, possibly the rate of PGA transport across the chloroplast envelope. The same suggestion has been made earlier by Day and Hatch (1981b) from

results obtained on uptake of ^{14}C -PGA by isolated maize mesophyll chloroplasts. If PGA reduction was not limited by non-cyclic electron transport but rather by translocator activity, the stimulation of PGA reduction by pyruvate could be an effect of the PEP formed inside the chloroplasts exchanging with external PGA. This would also imply that the stromal concentration of P_i limited the translocator activity.

The rate of substrate-dependent oxygen evolution with OAc as substrate (Tab. 3) was in the same range as with PGA. This reaction was also stimulated by pyruvate. This stimulation has similarly been interpreted to be due to uncoupling of non-cyclic electron flow (Kagawa and Hatch 1974, Day et al. 1981), which in this case seems more likely since OAc reduction only consumes NADPH.

The purified chloroplasts consumed oxygen in the light when no substrate or only pyruvate was present (Tab. 3). This reaction was obtained without the addition of a catalase inhibitor, which further establishes the purity of the preparation. The maximal rate of $19 \mu\text{mol O}_2$ consumed/mg Chl $\cdot$ h was lower than that reported by Huber and Edwards (1975c): $77 \mu\text{mol O}_2$ consumed/mg Chl $\cdot$ h (calculated from oxygen trace).

As mentioned in the introduction, published data on the ability of PEP to inhibit PGA-dependent O_2 evolution are in conflict with the proposal that PEP is transported on the general P_i -translocator, and not on a specific PEP/ P_i carrier. The results of our inhibition studies show that PEP inhibits PGA-dependent oxygen evolution if well purified chloroplasts are used. This confirms the proposal of Day and Hatch (1981b) that P_i , PGA and PEP share a common carrier similar to the P_i -translocator of C_3 chloroplasts. The stimulation of oxygen evolution obtained with protoplast extracts and PEP concentrations up to 12 mM (Fig. 3B) was probably due to OAc reduction rather than an increase in PGA reduction, since OAc would be formed from added PEP by PEP carboxylase in the cytosol-containing medium. We therefore believe that the conflicting results obtained with PEP have been due to PEP carboxylase contamination of the chloroplasts used.

In C_4 plants, P_i and PEP are major metabolites of the mesophyll chloroplasts, and both seem to be transported by the P_i -translocator across the chloroplast envelope. One may therefore expect that the K_m of the translocator would be relatively low for both compounds; in contrast to the situation in C_3 plants, where PEP is a minor chloroplast metabolite and the K_m for PEP is one order of magnitude higher than for P_i (Fliege et al. 1978). Uptake studies with $^{32}P_i$ and *Digitaria* mesophyll chloroplasts gave an apparent K_m for P_i of 0.2 mM and a K_i for PEP of 0.425 mM (Huber and Edwards 1977b). Similarly, the K_i 's for P_i and PEP were 0.6 and 1.5 mM, respectively, as measured by uptake of ^{14}C -PGA with maize mesophyll chloroplasts (Day and Hatch 1981b). Our results show that P_i and PEP are about equally efficient as inhibitors of PGA-dependent

O₂ evolution, which suggests that their K_m's for the P_i-translocator are similar. Thus, the results obtained so far indicate that P_i and PEP are both efficient substrates for the P_i-translocator in C₄ mesophyll chloroplasts. This would imply that the development from C₃ to C₄ metabolism also includes a change in the properties of the P_i-translocator of at least the chloroplasts located in the mesophyll cells.

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METABOLISM OF $[1-^{14}\text{C}]$ 3-PHOSPHOGLYCERATE WITH MESOPHYLL PROTO-
PLASTS AND PURIFIED MESOPHYLL CHLOROPLASTS FROM THE C_4 PLANT
DIGITARIA SANGUINALIS - FORMATION OF HEXOSE PHOSPHATES AND
STARCH¹

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Abbreviations used: PEP, phosphoenolpyruvate; OAc, oxaloacetate; 3-PGA, 3-phosphoglycerate; DHAP, dihydroxyacetonephosphate; RuBP ribulose-1,5-bisphosphate; FBP, fructose-1,6-bisphosphate; BSA, bovine serum albumine; Hepes, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)-aminomethane; HPI, hexosephosphate isomerase; G-1-P, glucose-1-phosphate; R-5-P, ribose-5-phosphate; F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; MAL, malate; ASP, aspartate; Glyc, glycerate; FBPase, fructose-1,6-bisphosphatase; PG mutase, phosphoglucomutase; Chl, chlorophyll.

Abstract

We have used isolated mesophyll protoplasts and highly purified mesophyll chloroplasts from the C_4 plant *Digitaria sanguinalis*, to reinvestigate their metabolism of 3-phosphoglycerate.

Protoplast extracts and chloroplasts metabolized ^{14}C labelled phosphoglycerate mainly to dihydroxyacetonephosphate, but label was also found in hexosephosphates and in starch; and with protoplast extracts also in the CO_2 fixation products malate, aspartate and oxaloacetate. Starch constituted up to 20 % of the label recovered inside the chloroplasts after 25 min incubation in the light (equal to $0.3 \mu\text{mol } ^{14}C$ incorporated/mg Chl $\times$ h). With pyruvate present, conversion of labelled phosphoglycerate was inhibited with protoplast extracts, indicating a competition for available NADPH between phosphoglycerate and oxaloacetate reduction.

The enzymatic capacity of mesophyll protoplasts and chloroplasts to form starch from triosephosphates was studied by assaying the following enzymes: aldolase, fructose-1,6-bisphosphatase, hexosephosphate isomerase, phosphoglucomutase and ADP-glucose pyrophosphorylase. Only low activities of aldolase and FBPase were found in both chloroplasts and protoplasts, whereas considerably higher levels of the other three enzymes were detected. One third to half of the activity of the first four enzymes was present in the mesophyll chloroplasts, as compared to the intact protoplasts. ADP-glucose pyrophosphorylase appeared to be a chloroplastic enzyme solely.

We conclude that mesophyll chloroplasts from *Digitaria* can form starch from phosphoglycerate, but that the rate may be several fold higher if hexoses are used as precursors for starch synthesis.

Introduction

C_4 plants are characterized by a division of labor between two different cell types. Atmospheric CO_2 entering the leaf is first fixed in the mesophyll cell cytoplasm by the highly efficient PEP-carboxylase. The OAc formed is reduced to malate in the mesophyll chloroplasts or aminated to aspartate either in the cytoplasm or in the chloroplasts. The C_4 acid (either malate or aspartate) is transported to the bundle sheath cell, decarboxylated, and the released CO_2 refixed via the Calvin cycle in the bundle sheath chloroplasts. Precursors for the regeneration of PEP are transported back to the mesophyll cell thus completing the cycle. (For a review see Hatch and Osmond (1)).

In *Digitaria sanguinalis*, a plant belonging to the NADP-malic enzyme group (2), malate is the main C_4 acid transported and pyruvate is returned to the mesophyll cell. Apart from performing C_4 acid converting reactions, the mesophyll chloroplasts also contain the enzymes of the Calvin cycle for converting PGA to DHAP (1,3). The levels of photosystem II in bundle sheath chloroplasts of NADP-malic enzyme type C_4 plants, have been shown to be low (connected with a low amount of grana in these chloroplasts) (4), leading to NADPH deficit. The decarboxylating reaction via NADP-malic enzyme can supply half of the NADPH needed for CO_2 fixation in the bundle sheath chloroplasts. The rest of the NADPH needed for the reduction of PGA to DHAP in the bundle sheath cell can come from the mesophyll chloroplasts via a shuttle of PGA and DHAP between the two cell types.

In the general scheme for C_4 photosynthesis the mesophyll chloroplasts do not perform the reactions of the Calvin cycle involved in the conversion of DHAP to sugar phosphates and to RuBP.

Early studies on enzyme compartmentation in C_4 plants performed on mechanically isolated mesophyll and bundle sheath cells, showed a clear preferential distribution of PEP-carboxylase to mesophyll cells and RuBP-carboxylase to bundle sheath cells in *Digitaria sanguinalis* (5) and in *Zea Mays* (6). However, these studies were hampered by the difficulty to obtain 100 % pure mesophyll cells, and enzymes showing low activity in these cells could be accounted for by the contamination from bundle sheath cells. Edwards and Black (5) found FBP aldolase in mesophyll cells from *Digitaria sanguinalis* but of the same magnitude as the contamination by bundle sheath material (as malic enzyme and RuBP carboxylase). Hatch and Kagawa (6) detected FBP aldolase and FB Pase in mesophyll extracts from *Zea mays* but at levels comparable to RuBP carboxylase and Ru-5-P-kinase.

By using enzymatically isolated mesophyll protoplasts a much purer mesophyll fraction was obtained (7,8 and Edwards and Huber (9) for a review). Studies with isolated protoplasts and chloroplasts from these protoplasts have confirmed earlier proposals, and ascertained the intracellular distribution of enzymes of the C_4 pathway and the PGA reducing complement of the Calvin cycle in the mesophyll cell, according to the general scheme mentioned above (see Hatch and Osmond (1) for references).

The occurrence of large amounts of starch in the bundle sheath chloroplasts (10,11) have also been taken as evidence supporting

the localization of the Calvin cycle in these cells (1). However, starch grains are also seen in mesophyll chloroplasts of *Zea mays* (10) and *Digitaria sanguinalis* (11). In the current scheme for carbon flow, starch can not be formed from DHAP in the mesophyll chloroplast. Instead Hatch and Osmond (1) proposed that other intermediates for starch synthesis were transported from bundle sheath to mesophyll cells. Kagawa and Hatch (12) studied the incorporation of label into starch with maize mesophyll chloroplasts when they were supplied with labelled PGA, glucose or glucose-1-P. Label in starch was negligible with these substrates and DHAP was the main labelled product formed from 3-PGA.

We have obtained pure mesophyll protoplasts from *Digitaria sanguinalis* using enzymatic digestion and partition in a polymer two-phase system. These protoplasts were used to prepare intact mesophyll chloroplasts with low cytoplasmic contamination (13). In this paper we report on the light-dependent metabolism of ^{14}C labelled 3-PGA with mesophyll protoplast extracts and purified chloroplasts, and on the activities of enzymes involved in the synthesis of starch from triose phosphates.

Materials and Methods

Materials

Dextran T500 and Percoll were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Polyethylene glycol 4000 (Carbowax 4000, recently renamed as Carbowax 3350) was from Union Carbide, New York. $\text{NaH } ^{14}\text{CO}_3$ (45.7 Ci/mol, 1.69 TBq/mol) was from New England Nuclear, Dreieich, W. Germany. ATP monitoring reagent was from LKB-Wallac, Turku, Finland. Other biochemicals, substrates and enzymes were from the Sigma Chemical Co.

Crabgrass (*Digitaria sanguinalis* (L.) Scop.) was cultured in a growth chamber under artificial light as previously described (13). Spinach (*Spinacia oleracea* L. cv. Viking II) was grown as in (14).

Synthesis of (1- ^{14}C) 3-PGA

Labelled 3-PGA (22.8 Ci/mol) was formed from RuBP and $\text{NaH } ^{14}\text{CO}_3$ by purified spinach RuBP carboxylase. Analysis by thin layer electrophoresis and chromatography (see below) showed that 94.1 % of the label was in ^{14}C -PGA, 5.1 % was an unidentified component with high electrophoretic mobility, 0.3 % was glycerate and 0.5 % other unidentified compounds

Preparation of *Digitaria mesophyll* protoplasts and chloroplasts

Mesophyll protoplasts were prepared from handcut segments from 16-18 days old leaves by enzymatic digestion, and purified by centrifugation and by partition in a dextran-polyethylene glycol two-phase system as described earlier (13,15). Intact mesophyll

chloroplasts, with low cytoplasmic contamination, were prepared from protoplasts according to (13), by gentle breakage of protoplasts, collection of chloroplasts by differential centrifugation, purification of chloroplasts by two-phase partition, and centrifugation through a Percoll cushion. Protoplasts and chloroplasts were stored at room temperature and on ice, respectively, in the assay medium: 0.4 M sorbitol, 50 mM Hepes-KOH (pH 7.6), 0.5 mM KH_2PO_4 and 0.1 % w/v BSA (2.5 mM P_i for protoplasts).

Protoplast extract

Intact protoplasts were passed through a 100- μl Hamilton syringe to produce a protoplast extract (3) which was used immediately.

Intact spinach chloroplasts

Chloroplasts were prepared from 6-8 weeks old deribbed leaves according to (14).

$^{14}\text{CO}_2$ fixation

The incorporation of $^{14}\text{CO}_2$ with protoplast extracts were studied at 30°C in assay medium including 2 mM EDTA and 2 mM potassium phosphate and with 7 μg Chl/100 μl . After 2 minutes preillumination in the presence of 2 mM 3-PGA reactions were started by the addition of 3.4 mM $\text{NaH}^{14}\text{CO}_3$ and 3 mM MgCl_2 . Reactions were stopped after 15 min by adding 50 μl 1 M HCl containing 0.6 % phenylhydrazine. Light (2500 $\mu\text{E m}^{-2}\text{s}^{-1}$ at 400 to 700 nm) was provided by a slide projector. Total incorporation of

^{14}C was determined by liquid scintillation counting of 5 μl samples spotted on 1 cm filter disks.

Conversion of (1- ^{14}C)3-PGA

Experiments with labelled 3-PGA were run in a total volume of 100 μl assay medium with 2 mM (1- ^{14}C)3-PGA and with 5-15 μg Chl/100 μl , at 30°C. Light was provided by a slide projector. With protoplast extracts the assay medium included 2 mM EDTA, 2 mM P_i , 3.4 mM NaHCO_3 and 3 mM MgCl_2 , and reactions were started as for $^{14}\text{CO}_2$ fixation. With *Digitaria* mesophyll chloroplasts the assay medium included 2 mM EDTA and 550 units of catalase. Reactions were started by turning on the light.

For all assays the reactions were terminated by addition of HCl to 0.33 M final concentration; with protoplast extracts by adding 50 μl 1 M HCl; with chloroplasts the distribution of labelled conversion products between chloroplasts and the surrounding medium was studied by transferring 90 μl of the 100- μl reaction mixture to a 1-ml centrifugal tube and pelleting the chloroplasts for 5 s in a Beckman Microfuge B. From the supernatant, 70 μl was added to 35 μl 1 M HCl, and then 50 μl of 0.33 M HCl was added to the well drained pellet. Total label in each fraction was determined as under $^{14}\text{CO}_2$ fixation.

Analysis of $^{14}\text{CO}_2$ fixation products

Using total acidified extract soluble and insoluble products of $^{14}\text{CO}_2$ fixation were separated by thin layer electrophoresis for 60 min, using conditions according to (16), and quantified by liquid scintillation counting as in (15).

Analysis of (1-¹⁴C)-PGA conversion products

Total products of 3-PGA metabolism were separated by thin layer electrophoresis and chromatography according to (16), followed by autoradiography. Radioactive spots were scraped off and determined with liquid scintillation counting. Identification was as in (16,17).

To check the starch contents in the radioactive spots remaining at the application points after the two dimensional separation, two supernatant and two pellet fractions from experiments with chloroplasts were analysed for labelled starch. Insolubles in 0.33 M HCl were pelleted in a Beckman Microfug B in 1 ml tubes. The pellets were washed five times with 0.5 ml water to remove all soluble radioactivity and the pellets were then treated with 6 M HCl at 60°C for 24 h to hydrolyze the starch. The HCl was evaporated at 60°C and the residue dissolved in 20 µl water. Glucose was identified after chromatography of the dissolved fractions on kieselgel 60 (E. Merck, Darmstadt) pretreated with 0.01 M sodiumacetate and developed twice with acetone-water (90:10). Radioactive compounds were located by autoradiography as above.

The supernatant fractions contained no detectable glucose; activity remained at the application point, and corresponded to 1 and 2.6 % of total calculated activity in the start spot of the original two-dimensional separation (calculated as relative amount in start spot times the activity in the sample used for starch analysis). With the two pellet fractions 70 % of the radioactivity cochromatographed with glucose, and the recovery was 18 and 44 % respectively, as calculated from the radioactivity

remaining at the start spot of the original two-dimensional separation. This means that at least 13 and 31 %, respectively, of the initial label in the start spots of the pellet fractions was starch in these two experiments.

Assay of enzymes

Aldolase (E.C. 4.1.2.13) was measured according to (18) in 80 mM triethanolamine, 20 mM EDTA (pH 7.5) also including 2 mM DTT and 0.05 % (v/v) Triton X-100. The chloroplasts or protoplasts were solubilized for 3 min and the baseline was recorded; FBP was then added to start the reaction. Fructose-1,6-bisphosphatase (E.C. 3.1.3. 11) was assayed in 100 mM Tris-HCl (pH 8.3), 1 mM EDTA, 10 mM MgCl_2 , 0.05 % (v/v) Triton X-100, 2 mM DTT containing 4 units glucosephosphate isomerase, 2 units glucose-6-phosphate dehydrogenase, 0.4 mM NADP^+ and 1 mM FBP (19). The baseline was recorded and the sample added; after 5 min of solubilization and enzyme activation, the activity was recorded. Hexosephosphate isomerase (E.C. 5.3.1.9) and phosphoglucomutase (E.C. 2.7.5.1) were measured at pH 8.0 and with 0.05 % (v/v) Triton X-100. essentially according to (6). A low background activity in the HPI assay was corrected for by measuring the baseline without sample; the sample was added and the activity recorded after 3 min of solubilization. In the phosphoglucomutase assay the baseline was recorded with solubilized sample and the reaction was started by adding G-1-P. All of the above assays were run in a final volume of 1 ml on an Amino Chance DW 2 spectrophotometer at 340 nm.

ADP glucose pyrophosphorylase (E.C. 2.7.7.b) was measured in the direction of ATP formation directly coupled to the light producing luciferase-ATP reaction. The assay was run in 0.1 M glycylglycin-KOH (pH 7.75), 2 mM PP_i and 0.8 mM 3-PGA. To 900 μ l buffer was added 100 μ l of the ATP monitoring reagent (re-constituted to 5 ml with water) which resulted in a final concentration of 10 mM Mg^{2+} and 0.1 % (w/v) BSA in the assay (according to the manufacturers specification). These concentrations were adopted from (20). The reaction was run in a 2 ml plastic tube and the light emission measured on a LKB-Wallac 1250 luminometer coupled to a chart recorder. After a baseline recording the reaction was started by adding 2 mM ADP-glucose. Light emission was calibrated by adding $1 \cdot 10^{-7}$ M ATP after 2 min. This addition gave a signal of 15-50 mV on the instrument as compared to about 1500 mV in an optimized system, which showed that the luciferase reaction was inhibited in the system used. However, the reaction was linear with time during the measurement and there was a linear response to consecutive additions of standard ATP up to 10^{-6} M. The samples were diluted 50 to 100 times with deionized water before use to get suitable enzyme activity. All five enzymes were assayed at 30°C with *Digitaria* material and at 20°C with spinach chloroplasts.

PGA-dependent oxygen evolution, chloroplast intactness and chlorophyll

Substrate dependent oxygen evolution was measured as described earlier (13). Chloroplast intactness was determined with the ferricyanide method (21) and chlorophyll was estimated according to Arnon (22).

Results

Mesophyll protoplasts prepared by us totally lack bundle sheath cell contamination, measured as R-5-P stimulated $^{14}\text{CO}_2$ fixation (15). Preparations of intact mesophyll chloroplasts, starting from these protoplasts, result in a chloroplast fraction with a cytoplasmic contamination of only 0.2-1 % of original protoplast activity, measured as PEP carboxylase activity (13). The chloroplasts used here had an intactness of 80 to 98 % and showed PGA-dependent oxygen evolution corresponding to about 100 μmol PGA reduced/mg Chl $\times$ h. The rate with protoplast extracts were between 100 and 180 μmol PGA reduced/mg Chl $\cdot$ h.

Metabolism of (1- ^{14}C)3-PGA and $^{14}\text{CO}_2$ fixation with protoplast extracts

The products of conversion of ^{14}C -PGA by mesophyll protoplasts. extracts are shown in Table I. The main product was DHAP, when ^{14}C -PGA was the only substrate (except for HCO_3^-), but label also appeared in hexose phosphates, in an unknown sugar phosphate (X in Table I, exp II), in PEP, and in the CO_2 fixation products OAc, malate and aspartate. PGA reduction and CO_2 fixation products constituted about 80 %, and 20 % of the label, respectively. To mimic the situation in vivo, with CO_2 fixation and PGA reduction running simultaneously, ^{14}C -PGA conversion was also studied in the presence of 2 mM pyruvate (Table I). In this case, the rate of ^{14}C -PGA conversion became inhibited by 30 to 70 % and the amount of label in PGA reduction products decreased by 40 to 80 %.

At the same time the label in CO_2 fixation products decreased by about 20 %.

In parallel experiments, the total $^{14}\text{CO}_2$ fixation rate was measured with the same substrates, using $\text{H}^{14}\text{CO}_3^-$ and unlabelled 3-PGA and pyruvate (Table II). The rates of $^{14}\text{CO}_2$ fixation with 3-PGA as substrate were comparable to those measured with ^{14}C -PGA. Malate and aspartate were the main products, together constituting 86 and 96 % of total labelled products in the two experiments shown. The addition of 2 mM pyruvate induced high rates of $^{14}\text{CO}_2$ fixation, with malate as the main product, and with only minor amounts of aspartate found. The rates of OAc reduction in the two experiments were 93 and 60 μmol OAc reduced/mg Chl $\cdot$ h respectively, as calculated from Table II.

Metabolism of (1- ^{14}C)3-PGA with purified chloroplasts

When chloroplasts were allowed to metabolize ^{14}C -PGA for 15 min, over 90 % of the label was found in DHAP outside the chloroplasts (Table III). The chloroplast pellet contained relatively more of hexose phosphates (25 %), as compared to the supernatant (3.6 %). The start spot from the supernatant fraction did not contain any detectable starch and the starch content in the pellet fraction was estimated to be maximum 70 % of the start spot (corresponding to 6.2 % of the total label in the pellet). Chloroplasts were also allowed to metabolize ^{14}C -PGA, (4 mM) for 25 min but the general pattern and distribution of products showed little difference, as compared to the 15 min incubation (Table III b). The relative amount of

label in starch in the chloroplast pellet increased to maximum 11 % and there was a markedly higher amount of unidentified products in the supernatant, 12.6 % of total label recovered. Attempts were made to stimulate PGA reduction by adding 2 mM pyruvate, which in the case of isolated chloroplasts would lead to an increased ATP consumption and thereby an uncoupling effect on PGA reduction. No increase in the rate of ^{14}C -PGA conversion was seen, and the distribution of label resembled that without pyruvate, except that the relative label in starch in the pellet had increased at the expense of hexose phosphates (not shown). An experiment was performed to mimic the case with protoplast extracts plus pyruvate, by directly supplying purified chloroplasts with 2 mM OAc during ^{14}C -PGA conversion. Also in this case the differences were small as compared to the results without OAc, both regarding ^{14}C -PGA conversion rate and distribution of label (not shown). The expected inhibition of PGA reduction by OAc reduction due to competition for NADPH was therefore not observed with isolated chloroplasts.

Enzymatic capacity for starch formation

The activities of enzymes needed for formation of starch (ADP-glucose) from DHAP were studied in intact mesophyll protoplasts and isolated chloroplasts. For comparison with chloroplasts from a C_3 plant containing the Calvin Cycle as well as starch synthesis, these enzymes were also measured in isolated spinach chloroplasts (Table IV).

All enzymes assayed were present in *Digitaria* mesophyll chloroplasts. The specific activities of aldolase and FBPase

were low as compared to spinach chloroplasts. HPI, PG mutase and ADP-glucose pyrophosphorylase showed equal or higher specific activities in *Digitaria* mesophyll chloroplasts compared to spinach chloroplasts.

When the specific activities in the intact protoplasts are compared with those in the isolated mesophyll chloroplasts, it can be seen that one third to half of the activity of the first four enzymes was associated with the chloroplasts. Chloroplasts and protoplasts had the same specific activity of ADP-glucose pyrophosphorylase.

Discussion

Based on enzyme compartmentation studies and on measured activities, the mesophyll chloroplasts of C_4 plants have been ascribed three main metabolic functions in the C_4 pathway of photosynthesis: Formation of PEP from pyruvate; reduction of OAc to malate; and conversion of PGA to DHAP. To be able to make a clear cut study of the capacity and localization of metabolic steps in a pathway, and connect these to a specific organelle, one must use extremely well defined fractions. We have reported on the preparation of highly purified intact mesophyll chloroplasts from *Digitaria sanguinalis* (15), with low cytoplasmic contamination. Such chloroplasts have been used for this study.

Metabolism of labelled 3-PGA

The results from the conversion of ^{14}C -PGA by mesophyll protoplast extracts and isolated chloroplasts (Table I, III), with DHAP as the main product, are in agreement with the expected enzymatic capacity of these chloroplasts. However, we also found label in hexose phosphates and in starch. These results are partly in conflict with those of Kagawa and Hatch (12) who could not detect metabolites beyond DHAP with maize mesophyll chloroplasts (mechanically isolated, washed once).

With protoplast extracts around 20 % of the basal rate of PGA conversion was to PEP formation and concomitant CO_2 fixation; PGA can be converted to PEP by cytoplasmic phosphoglyceromutase and enolase, both enzymes present in *Digitaria* mesophyll protoplasts (23).

Since the reduction of OAc to malate consumes NADPH, this reaction can compete with PGA reduction for available NADPH. This competition was studied by adding pyruvate, which would be metabolized to PEP and further to OAc. Under these conditions, the rate of PGA reduction, as well as the total rate of PGA conversion, was reduced by 30 to 70 % with protoplast extracts (Table I). At the same time the rate of CO_2 fixation from labelled 3-PGA was almost unaffected. A similar result was obtained earlier with intact protoplasts (24). The addition of pyruvate stimulated the rate of $^{14}\text{CO}_2$ fixation with unlabelled PGA as substrate, and the main labelled product was malate (Table II). The rate of OAc reduction in this case was apparently high enough to compete with PGA reduction for NADPH. The stimulation of $^{14}\text{CO}_2$ fixation by pyruvate is an effect of an increased PEP formation via the pyruvate-Pi-dikinase enzyme in the chloroplasts. The formation of 1 molecule of PEP consumes 2 ATP and thereby uncouples noncyclic electron transport leading to the observed increased capacity for OAc reduction.

The distribution of labelled products between the medium and chloroplasts were studied by simply separating the particles from the solution by a single centrifugation. Most of the labelled conversion products appeared as DHAP outside the chloroplasts as would be expected from the predicted metabolic role of the mesophyll chloroplast - import of PGA and export of DHAP. A general feature of the experiments presented was that the absolute amount of labelled sugar phosphates always was greater in the supernatant than in the pellet. The external

sugar phosphates could either be due to leakage from or breakage of chloroplasts during the measurement or to contaminating stromal enzymes outside the chloroplasts, forming hexose phosphates from exported triosephosphates. Since hexose phosphates are not transported by C_3 chloroplasts (25) and the chloroplasts have been shown to be highly intact and metabolically competent (13), we consider enzyme contamination to be the main reason for the occurrence of hexose phosphates outside the chloroplasts. A similar contamination phenomenon leading to formation of FBP from DHAP has been described for spinach chloroplasts (14).

Protoplast extracts showed inhibition of PGA reduction when pyruvate was added (leading to OAc reduction). The lack of effects of OAc on the rate of PGA conversion with isolated chloroplasts can be explained by the possibly translocator activity limited rate of PGA reduction proposed for these chloroplasts (13). Thus, the addition of OAc would lead to an increased non cyclic electron transport and NADPH production, and a reduction of OAc to malate, without affecting the rate of PGA reduction. This was seen as an increased rate of O_2 production (13).

The stimulation of PGA-dependent oxygen evolution by pyruvate with maize mesophyll chloroplasts (26) and *Digitaria* mesophyll chloroplasts (1.5 fold) (13) has been interpreted as an increased PGA reduction due to an uncoupling effect on non cyclic electron transport, or to an increased translocator activity; the PEP formed from pyruvate could exchange with PGA. The predicted increase in ^{14}C -PGA conversion (reduction) was

however not observed in these experiments, which is difficult to explain. Simultaneous measurements of O_2 evolution and ^{14}C -PGA reduction has not been performed, but supposing that oxygen production directly reflects the rate of substrate turnover, the results with pyruvate and labelled PGA are in conflict with those obtained during oxygen evolution measurements (13). Kagawa and Hatch (12) found an increased production of DHAP with *Atriplex* mesophyll chloroplasts, when the chloroplasts were supplied with pyruvate during PGA reduction. Day et al. (26) observed a stimulation by pyruvate during PGA-dependent oxygen evolution with maize mesophyll chloroplasts, but they did not analyze the production of DHAP.

Enzymes for synthesis of starch

The route to starch from triosephosphates involves the formation of FBP by FBP aldolase, conversion of FBP to F-6-P by FBPase; these two enzymes being part of the Calvin cycle. F-6-P is converted to G-6-P by hexosephosphate isomerase and phosphoglucomutase, respectively; G-1-P plus ATP yields ADP-glucose by the action of ADP-glucose pyrophosphorylase. ADP-glucose is then used by the enzyme starch synthetase to couple the glucose residue to the starch molecule. Aldolase and HP isomerase can also be part of glycolysis and FBPase of the glycogenesis. Phosphoglucomutase and ADP-glucose pyrophosphorylase are unique enzymes of the starch synthesis; the mutase also takes part in the degradation of starch.

If the activities of these five enzymes in isolated mesophyll protoplasts and purified chloroplasts from *Digitaria*, are compared with those of isolated spinach chloroplasts, two

important features emerge: (1) The capacity for synthesis of F-6-P from triosephosphates in *Digitaria* was low, and (2) the capacity to form ADP-glucose from F-6-P was equal or higher than in spinach.

The activities of the two Calvin cycle enzymes in mesophyll chloroplasts were above the calculated rate of incorporation of label into the starch fraction in Table III a,b (0.025-0.045 μmol hexose incorporated into starch/mg Chl $\times$ h compared to 1.6 according to the enzyme measurement). We consider these enzymatic activities to be a result of an inherent metabolic activity and not of a contamination by bundle sheath material, since our preparation of *Digitaria* mesophyll material does not show any R-5-P stimulated $^{14}\text{CO}_2$ fixation (13). Activities of aldolase and FBPase reported by others for mesophyll cells (see introduction) were of the same magnitude as the contamination by bundle sheath material. However, Raghavendra and Das found 38 and 27 % of the total leaf activity of aldolase and FBPase, respectively, in mechanically prepared mesophyll cells from *Setaria italica* (NADP-ME type) (27). These mesophyll cells were contaminated with 5 % of the total NADP-malic enzyme activity and 1.6 % of the total RuBP carboxylase activity.

Isolated *Digitaria* mesophyll chloroplasts and spinach chloroplasts showed comparable activity of HP isomerase (Table IV). Hatch and Kagawa (6) obtained activities of this enzyme in maize mesophyll cells far beyond the contamination level, but the isolated chloroplasts contained almost no activity.

PG mutase was present in *Digitaria* mesophyll chloroplasts with higher activities than in spinach. Hatch and Kagawa (6) obtained results with this enzyme similar to their results with the

HP isomerase.

Our results with ADP-glucose pyrophosphorylase showed that this enzyme was in the mesophyll chloroplast in *Digitaria*. In an earlier study on this plant by Edwards and Black (5), the presence of starch phosphorylase in mesophyll cells was clearly shown. However, the enzyme for synthesis of starch from ADP-glucose was not detected in mesophyll cells, and the authors raised doubts about the capacity for starch synthesis in these cells. Downton and Hawker (28), working with mechanically isolated maize mesophyll cells, found ADP-glucose pyrophosphorylase and ADP-glucose starch synthetase corresponding to 10 and 16 % of the specific activity in the bundle sheath cells, respectively. The bundle sheath contamination in their preparation was 10 % (malic enzyme).

Conclusions

Based on the results presented in this paper we propose that mesophyll chloroplasts of the NADP-malic enzyme type C_4 plant *Digitaria sanguinalis* contain the enzymes necessary for synthesis of starch from triosephosphates. The measured limiting enzymatic capacity (aldolase) corresponds to around 10 μ atoms carbon incorporated/mg Chl x h; and label from ^{14}C -PGA was incorporated into starch at a rate corresponding to about 0.3 μ atom ^{14}C /mg Chl x h with isolated chloroplasts.

However, the capacity of the mesophyll chloroplasts to form starch from hexose phosphates are considerably higher and comparable to that of C_3 chloroplasts. Hence, under conditions when starch synthesis is favoured in mesophyll chloroplasts, hexoses exported from the bundle sheath cell, rather than PGA, may be used as precursors to deposit starch in the mesophyll cells.

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TABLE I

PRODUCTS OF $[1-^{14}\text{C}]$ 3-PGA CONVERSION BY MESOPHYLL PROTOPLAST
EXTRACTS

Distribution of label after 15 min of ^{14}C -PGA conversion^a

Product	Additional substrate			
	None		2mM Pyruvate	
	I ^b	II ^b	I	II
DHAP	16130	11525	10156	1265
F6P ^c	765	728	305	38
FBP ^c	574	222	300	44
G6P ^c	752		306	35
X		1260 ^d		1060
PGA reduction	(20300)	(14700)	(12000)	(2558)
OAc	250		198	129
PEP	232	1571	937	1254
MAL	2451	693	2474	1292
ASP	1505	1267	75	21
CO ₂ fixation	(4440)	(3500)	(3700)	(2700)
Glyc	130	570	314	548
Others	2.3	4.3	6.0	8.0
START ^e	32	34	69	201
^{14}C -PGA conversion	102	80.3	68.2	26.1
μmol/mgChlxh				
(% of added)	(88)	(66)	(59)	(22)

^aThe radioactivity in different products is expressed as nmol/mgChl except for others which is given as percentage. The amount of ^{14}C -PGA metabolized through PGA reduction and CO₂ fixation, respectively is summed (within brackets).

^bFigures are from two preparations, I and II.

^cThese are calculated using twice the specific activity of the labelled 3-PGA.

^dIncludes G6P.

^eStarch content in Start spot: see Materials and Methods.

TABLE II

PRODUCTS OF $^{14}\text{CO}_2$ FIXATION WITH MESOPHYLL PROTOPLAST EXTRACTSDistribution of label after 15 min of $^{14}\text{CO}_2$ fixation^a

Product	Substrate			
	2 mM PGA		2mM PGA + 2mM Pyruvate	
	I ^b	II ^b	I	II
Malate	69.9	18.6	86.6	79.2
Aspartate	26.2	66.5	3.6	9.6
OAc	1.0	4.1	6.0	8.2
Others	2.8	10.8	3.8	3.0
$^{14}\text{CO}_2$ fixation μmol/mgChlxh	18.0	24.1	107	76.6

^aFigures are percentage of total fixed $^{14}\text{CO}_2$.^bResults are given for two preparations, the same as in Table I.

TABLE III

PRODUCTS OF $[1-^{14}\text{C}]$ 3-PGA CONVERSION BY PURIFIED MESOPHYLL
CHLOROPLASTS

(A) Distribution of label after 15 min of ^{14}C -PGA conversion ^a		
Product	Supernatant ^b	Pellet ^b
DHAP	9900 (92.5)	127 (63.0)
F6P	48 (0.89)	6 (6.2)
FBP	92 (1.7)	2 (2.4)
G6P	54 (1.0)	16 (16.4)
X ^c	(0.3)	
Others	(2.8)	(3.2)
START ^d	30 (0.28)	18 (8.8)
Total	98.2	1.8
(%)		
^{14}C -PGA converted		44.1
$\mu\text{mol/mgChlxh}$		
(% of added)		(87.1)

(B) Distribution of label after 25 min of ^{14}C -PGA conversion^{a,e}

Product	Supernatant ^b	Pellet ^b
DHAP	18200 (85.2)	184 (57.6)
F6P		4 (2.7)
FBP	64 (0.60)	15 (9.3)
G6P	53 (0.50)	12 (7.6)
X ^c	(0.46)	
Glyc	124 (0.58)	1 (0.33)
Others	(12.8)	(6.3)
START ^d	58 (0.27)	52 (16.2)
Total	98.5	1.5
(%)		
^{14}C -PGA converted	52.5	
$\mu\text{mol/mgChl}\cdot\text{h}$		
(% of added)	(85.3)	

^aAmount of products is expressed as nmol/mgChl or as percentage of supernatant and pellet respectively (within brackets).

^bChloroplasts were separated from the assay solution by a short centrifugation before killing and analysis.

^cUnknown phosphate containing carbon compound.

^dStarch content in START spot: Supernatant contained no starch; Pellet start spot contained maximal 70 % as starch (see Materials and Methods).

^e ^{14}C -PGA was 4 mM in the 25 min incubation.

TABLE IV

ENZYME ACTIVITIES IN *D. sanguinalis* MESOPHYLL PROTOPLASTS,
PURIFIED MESOPHYLL CHLOROPLASTS AND IN ISOLATED SPINACH
CHLOROPLASTS

Enzyme ^a	Specific activity ^b		
	Digitaria Protoplasts	Digitaria Chloroplasts	Spinach Chloroplasts
I Aldolase	8.8 ± 1.5	3.2 ± 0.1	148 ± 43
II FBPase	29.4 ± 8.6	10.6 ± 4.8	44 ± 38
III HPI	143 ± 70	40.6 ± 4.0	33.1 ± 4.2
IV PGmutase	82 ± 19	36 ± 16	16.8 ± 6.5
V ADP-G pyro- phosphorylase	70 ± 27	66 ± 16	50.2 ± 9.4 ^c

^aEnzymes are FBPase, fructose-1,6-bisphosphatase; HPI, hexosephosphate isomerase; PG mutase, phosphoglucomutase; ADP-G, ADP-glucose (pyrophosphorylase).

^bFigures are μmol substrate utilized or product formed/mgChlxh (I, NADH; II-IV, NADP; V, ATP formed). Mean $\pm$ SD, n = 4 are given.

^cn = 3.

